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in Breast Epithelial Cells

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Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that localizes to focal adhesion. FAK works as a transducer of extracellular signals that enter the cell via the integrins. FAK has been found to play an important role in normal biological process, including focal adhesion turnover, spreading, motility, cell cycle progression and cell survival. Many of the cellular processes mediated by FAK are deregulated in cancer, including proliferation, survival, as well as motility and invasion, which are key steps in metastasis. FAK is found to be overexpressed in a variety of cancer cells and tumors, including breast cancer tumors. Furthermore, FAK expression correlates with highly motile cancer cells and tumor invasiveness. Since FAK is overexpressed in breast cancer, and it is a mediator of cell cycle progression, motility and survival, the role of increased FAK signaling in progression and/or acquisition of cancer phenotypes will be investigated. A breast cancer cell model system will be used to increase FAK signaling, by overexpression of wild type FAK or an activated FAK construct, SuperFAK, in normal breast epithelial cells. Conversely FAK signaling will be inhibited by expressing of a naturally occurring dominant negative FAK variant, FRNK, in breast cancer epithelial cells.

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INTRODUCTION

The focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that localizes to focal adhesions (FA) (1;2). FAK is activated upon integrin-mediated cell adhesion (2-5) and works as a transducer of extracellular signals that enter the cell via the integrins, cell surface ECM receptors (6-9). FAK can also be activated by treating cells with a variety of soluble factors (10-17). FAK functions in normal biological processes including focal adhesion turnover (18), spreading (8), motility (18-20), cell cycle progression (19;21), and cell survival (19;22;23). Many of the cellular processes mediated by FAK are deregulated in cancer, including proliferation, survival, as well as motility and invasion, which are key steps of metastasis. Elevated Src activity has been observed in human cancers, including breast cancer (24;25). Interestingly, FAK was first identified in Src transformed cells (26) and Src is known to bind and phosphorylate FAK (27-30). These observations suggest a role for FAK in oncogenic transformation. FAK itself is overexpressed in a variety of cancer cells (31-33) and tumors (34-36), including breast cancer tumors (37;38). Furthermore, elevated FAK expression correlates with highly motile cancer cells and tumor invasiveness (37;39;40), suggesting a role for FAK in the progression of cancer cells to a more invasive phenotype. Since FAK is overexpressed in breast cancer, and it is a mediator of cell cycle progression, motility, and survival we decided to study the role of increased FAK signaling in the progression and/or acquisition of breast cancer phenotypes. A cell model system will be used to increase FAK signaling, by overexpression of wild type or a constitutively activated FAK construct, SuperFAK, in normal breast epithelial cells. Conversely FAK signaling will be inhibited through the expression of a naturally occurring dominant negative FAK variant, FRNK (41;42), in cancer breast epithelial cells.

BODY

In order to increase FAK signaling in breast epithelial cells, we proposed to construct activated mutants of FAK. Appendix B contains a manuscript in preparation. The manuscript describes the construction and biochemical as well as biological characterization of SuperFAK and FAK6.7, two activated mutants of FAK. The biochemical studies were performed in chicken embryo fibroblasts, since this was the cell line in which FAK was first identified and characterized (1). Most importantly, the use of the avian replication competent retroviral vectors in the CEF expression systems, (1) allows for high efficiency of expression and thus has become a useful and successful system for biochemical analysis. Compared to wild type FAK, SuperFAK, and to a lesser degree FAK6.7, show increase catalytic activity (Appendix B; Figure 2 and 4), which leads to a correlative increase in downstream FAK signaling, as demonstrated by increased tyrosine phosphorylation of FAK substrates (Appendix B; Figure 3 and 4). Of further importance and relevance to this project is the fact that SuperFAK can further increase the motility of T47D cells compared to wild type FAK (Appendix A; Figure 8). The studies described in the manuscript demonstrate that SuperFAK is a powerful tool that can be used to increase the biochemical and biological signals mediated by FAK.

The next step we proposed was to express FAK, SuperFAK and FRNK, a naturally occurring dominant negative of FAK (41;42) in normal immortalized and cancerous human epithelial cells, MCF10A and T47D respectively (44;43). The constructs of interest were all cloned in an avian replication competent retroviral vector, RCAS type A, for expression and characterization in CEF. Upon successful transfections

into CEF, the DNA of interest inserted as well as genes that encode for an avian replication competent retrovirus are inserted in the cell's genome (Appendix A; Fig 1A). The CEF cells can thus produce retrovirus carrying the RNA for the constructs of interest. This avian virus can in turn infect other avian cells by binding to the corresponding avian retroviral receptor, Tva (Appendix A; Fig 1A). However, the virus will not be able to infect cells from other species. In collaboration with the laboratory of Dr. L. Parise at the University of North Carolina at Chapel Hill, we were able to stably express the avian retroviral receptor, Tva, in T47D cells. Virus collected from CEF expressing wild type FAK, the activated constructs, SuperFAK or FAK6.7, or FRNK, was used to infect the T47D/Tva cells. Ten-14 days after infection of the T47D/Tva cells were lysed and expression of FAK examined by Western blotting. At first we had difficulty expressing high levels of exogenous protein, but by titrating the amount of virus added to the T47D/Tva cells we were able to achieve maximal expression (Appendix A; Fig 1B and Fig 6B). Thus, we have devised a cell system in which we can successfully express the FAK constructs.

Since the T47D/Tva system had been so successful, we decided to stably express the retroviral receptor, Tva, in the MCF10A cells. Unfortunately, none of the clones isolated after the antibiotic selection process expressed the Tva receptor. We also tried to derive MCF10A cells stably expressing FAK constructs driven by the CMV promoter. However, these efforts were also unsuccessful. The third strategy for expression was to subclone the FAK constructs into mammalian retroviral vectors and use packaging cell lines to make amphotrophic retrovirus (Appendix A; See Fig 2A) (45;46). The FAK, SuperFAK, and FAK6.7 were cloned into two different retroviral vectors (pBABE or pLPCX), transfected into the packaging cells. The packaging cell lines (Phoenix NA or 293GPG) (45;46) are cells that stably express in their genome the genes needed for packaging RNA into retroviral particles. Retroviral particles containing the FAK constructs were collected and used for infection of cells. In addition to using the normal human breast epithelial cell line, MCF10A, we also decided to use a normal mouse breast epithelial cell line, HC11, to study the effect of increased FAK signaling in the acquisition of cancerous phenotypes. FAK Western blotting of MCF10A and HC11 cells infected with the retrovirus produced in the 293-GPG and Phoenix packaging cells demonstrated successful expression of FAK, SuperFAK (Appendix A; Fig 2B) and FAK6.7 (Appendix A; Fig 2C). Although more work has to be done in order to obtain the most favorable condition for maximal expression, these studies demonstrate that this retroviral system is a fast and effective way to express the FAK constructs in the breast epithelial cells.

The next step is to make sure that SuperFAK, FAK6.7 and FRNK expressed in the epithelial cells are behaving as expected. A number of biochemical and biological assays were proposed for this purpose. The biochemical and biological characterization of SuperFAK and FAK6.7, as well as FRNK, in CEF already indicate the value of these constructs in elevating or inhibiting biochemical as well as biological signals mediated by FAK (Appendix A) (41;42). To measure catalytic activity of the activated constructs, wild type FAK and SuperFAK were immunoprecipitated from T47D/Tva expressing cells and subjected to *in vitro* kinase assays. SuperFAK had a higher catalytic activity *in vitro* than wild type FAK (Appendix A; Fig 3). This observation is similar to the results obtained when SuperFAK was expressed in CE cells (Appendix B). The ability of the

activated constructs to increase FAK downstream signaling in the breast epithelial cells was investigated by measuring the phosphotyrosine level of a FAK substrate, paxillin, from T47/Tva expressors or total cellular phosphotyrosine of MCF10A expressing cells. When SuperFAK was expressed, a slight increase in cellular phosphorylation in MCF10A and paxillin phosphorylation in T47D was observed compared to wild type FAK expressors (Appendix A; Fig 4A&B). FAK is known to mediate a motility response (18-20). To determine if FAK expression induces a biological response, the effect of the FAK constructs on the motility of the T47D/Tva cells was measured using a transwell system (Appendix A; Fig 5A). The expression of FAK in the T47D was enough to increase the motility of T47D cells in response to a haptotactic signal (Appendix A; Fig 5B). Furthermore, SuperFAK expressing T47D cells exhibited an increased motility compared to wild type FAK expressing cells. FAK6.7 actually inhibits motility, suggesting FAK6.7 will not be a good tool to increase FAK signaling. However, it could be a valuable tool for understanding FAK's mechanisms of action. Although FRNK has already been shown to inhibit motility, the effect of FRNK in T47D motility was also assessed. Preliminary experiments indicate a trend for FRNK to inhibit T47D motility (Appendix A; Fig 5B). Although additional testing is required, FAK, SuperFAK and FRNK seem to be potent tools for the activation or inhibition of FAK signaling respectively, in T47D cells. The T47D/Tva cells and the FAK constructs at hand appear to be a useful system that will allow the investigation of the role of FAK signaling in the acquisition of cancer phenotypes.

The ultimate goal of the proposed project is to study the potential role of FAK signaling in carcinogenesis. For this purpose, the investigation was divided into two parts. First, if FAK signaling is increased in normal breast epithelial cells, are cancer phenotypes acquired? Furthermore, does increasing FAK signaling in breast cancer cells create a more aggressive phenotype. One of the typical characteristics acquired by cells through transformation is an ability to grow in the absence of adhesion. Therefore, the ability of MCF10A expressing wild type FAK or the activated construct, SuperFAK, to form colonies in a soft agar was monitored as a measure of adhesion independent growth. No difference was observed in the number or size of colonies formed between MCF10A expressing empty vector, wild type FAK, or SuperFAK. These observations suggest that increased FAK signaling might not be the critical factor or the only factor required for acquisition of the adhesion independent growth phenotype. However, it is also likely that the levels of expression of FAK and SuperFAK were not sufficient to increase FAK signaling, which is corroborated by the slight increase in cellular phosphotyrosine content in MCF10A expressing SuperFAK or wild type FAK, compared to vector alone (Appendix A; Fig 4B). Future work will focus upon enhancing the yield of retrovirus production and efficiency of infection to maximize expression. In the case of breast cancer epithelial cells, the expression of FAK or SuperFAK had no significant effect in the number or size of colonies formed in soft agar. Thus, the expression of FAK or SuperFAK is not sufficient to increase adhesion independent growth of the T47D cells, which have already acquired the ability to grow in soft agar. These observations do not eliminate the possibility that increased FAK signaling might affect other phenotypes, like invasion, rendering the cells more aggressive. Further work is in progress to test this possibility.

The second part of the investigation will examine if inhibiting FAK signaling in breast cancer cells inhibits some phenotypes associated with cancer. For this purpose, FRNK, a dominant negative form of FAK, was expressed in T47D cells in order to inhibit FAK signaling. It was demonstrated by measuring T47D motility, that FRNK is able to inhibit FAK mediated biological signals (Appendix A; Fig 5B). T47D cells expressing FRNK were grown in soft agar, as a measure of adhesion independent growth. After 10 days, a dramatic decrease in the number of colonies formed was observed (Appendix A; Fig 6A). These observations indicate that by inhibiting FAK signaling some phenotypes associated with cancer can be impaired. Further studies are underway to measure the ability of FRNK to inhibit other phenotypes associated with transformation as well as its ability to inhibit tumor formation in animals, as proposed. FAK as been proposed to be able to mediate an adhesion dependent survival signal (19;22;23). Thus, FRNK might be inhibiting a survival signal in T47D cells, which in turn would cause cells to die, thus leading to a decrease in the number of colonies. If FRNK was causing the cells to die, it would lead to a selection against FRNK expressing cells. However, FRNK expression in the T47D cells was stable for up to 9 weeks after infection, indicating that there is no selection against FRNK expressors (Fig 6B). In addition, preliminary experiments indicate that the FRNK expressing T47D cells are not dying, as measured by an apoptosis assay (Fig 7). Considering the proposed role of FAK in cell cycle progression (19;21), experiments are underway to determine if the effect of FRNK on the adhesion independent growth of the T47D cells is mediated by an inhibition of T47D proliferation.

KEY RESEARCH ACCOMPLISHMENTS

TASK 1

- Construction of T47D/Tva system of expression
- Expression of wild type FAK, SuperFAK and FRNK in T47D/Tva cells
- Retroviral system setup for expression in MCF10A and HC11 normal breast epithelial cells.
- Expression of wild type FAK, SuperFAK, and FAK6.7 in MCF10A and HC11

TASK 2

- SuperFAK and FAK6.7 expressed in T47D/Tva have increased catalytic activity compared to wild type FAK (Kinase Assays)
- Increased motility of T47D/Tva cells upon FAK expression, and further elevation by SuperFAK expression.
- Inhibition of motility of T47D/Tva cells by FRNK expression

TASK 3

- Soft agar assays system set up for T47D and MCF10A cells
- Inhibition of T47D colony formation in soft agar system by FRNK
- Demonstrated that FRNK does not seem to have any effect on T47D cell viability
- Completion of Flow Cytometry Workshop at the Flow Cytometry Facility at the University of North Carolina at Chapel Hill
- Completion of "Mice: Basic Handling & Technique Workshop" at the Office of Institutional Animal Care and Use at the University of North Carolina at Chapel Hill

REPORTABLE OUTCOMES

APPENDIX A	Data figures
APPENDIX B	Manuscript in progress
APPENDIX C	Keystone Meeting Abstract
APPENDIX D	American Society for Cell Biology Meeting Abstract

CONCLUSIONS

The manuscript in progress indicates that we have successfully built an activated FAK form, SuperFAK. Although, further work is necessary to express wild type FAK and SuperFAK to higher levels in MCF10A and HC11 normal breast epithelial cells, SuperFAK is a valuable tool to study the effect of increased FAK signaling in the acquisition of cancer phenotypes in the normal breast epithelial cells and the progression to a more aggressive phenotype of breast cancer epithelial cells. The expression of FRNK in T47D cells leads to a decreased ability of the cells to grow in the absence of adhesion, one of the hallmarks of cancer. This suggests an important role of FAK signaling in acquiring cancer phenotypes, such as adhesion independent growth. Further work is underway to monitor the effect of FRNK, and thus FAK signaling, on the ability of the T47D cells to form tumors in mice. The potential role of FAK signaling in breast cancer acquisition or progression will make it an important target for breast cancer therapeutics.

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APPENDIX A

Annual Summary Report Figures

Figure 1. T47D Expression System. **A.** Wild type FAK, SuperFAK, FAK6.7 and FRNK were cloned into a vector encoding for a replication competent avian retrovirus. The constructs were transfected into chicken embryo fibroblasts (47). The resulting retrovirus produced by the CEF was collected. T47D cells stably expressing the avian retroviral receptor, Tva, were cultured with the virus. Ten to 14 days after infection, the expression of the FAK constructs was monitored. **B.** Lysates (25 μ g) from T47D/Tva cells infected with virus from CEF, were subjected to SDS-PAGE followed by Western blotting with a polyclonal antibody, BC4, for FAK. Mock represents T47D/Tva cells infected with retrovirus carrying empty vector. "1" and "2" represent the amount of virus in mls used. 1 ml of virus allowed for maximum expression.

Figure 2. MCF10A and HC11 Expression System. **A.** Wild type FAK, SuperFAK and FAK6.7 were cloned into a retroviral vector for expression in a packaging cell line (Phoenix NA or 293-GPG). The resulting amphotrophic retrovirus was used to infect MCF10A and HC11 normal breast epithelial cells. MCF10A (**B**) and HC11 (**C**) lysates (25 μ g) infected with the retrovirus from (**A**) were subjected to SDS-PAGE and then Western blotted with a polyclonal antibody, BC4, for FAK. Mock represents cells infected with virus carrying empty vector. **C.** Note that when 4 μ g of DNA was used to transfect the constructs into the packaging cell lines, better expression was achieved upon infection of HC11 compared to only 1 μ g.

Figure 3. T47D/Tva Kinase Assays. **A.** FAK constructs were immunoprecipitated from T47D/Tva cells expressing vector alone (mock), wild type FAK, or SuperFAK. After washing the immune complexes, the kinase reactions were performed by incubating the immune complexes in Kinase Buffer and 10 μ Ci of 32 P-ATP for the indicated times. The reactions were stopped with the addition of Laemmli sample buffer. Samples were boiled and denature, subject to SDS-PAGE and the 32 P incorporation was monitored by autoradiography. **B.** The immune complexes were also Western blotted for FAK to control that equal amounts of protein were present in every one of the reactions.

Figure 4. Phosphotyrosine Signaling. **A.** (Top Panel) Paxillin was immunoprecipitated from T47/Tva cells expressing vector alone (mock), FAK, SuperFAK, or FAK6.7. The immune complexes were washed and subjected to SDS-PAGE followed by Western blotting with a phosphotyrosine antibody. (Bottom Panel) Paxillin immune complexes were also Western blotted for paxillin to ensure that equal amount of protein were present in every one of the immunoprecipitates. **B.** Lysates (25 μ g) of MCF10A cells expressing equal levels of vector alone (mock), FAK or SuperFAK were subjected to SDS-PAGE and Western blotted for phosphotyrosine.

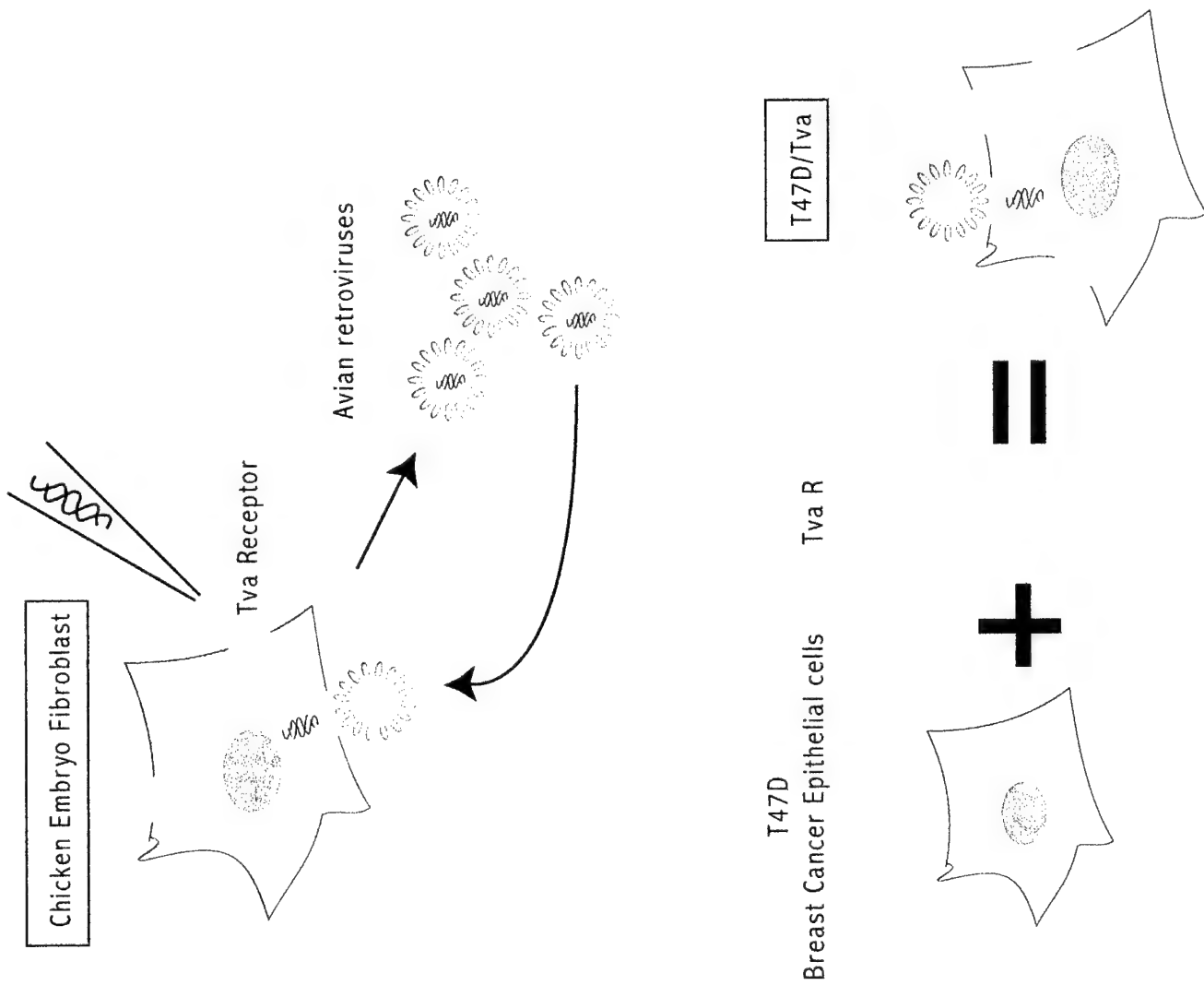
Figure 5. T47D Haptotactic Motility. **A.** In a transwell assay system, the underside of the transwell membrane was coated with 40 μ g/ml collagen I (6 hrs). The cells (150,000) were added to the top of the washed transwells. Motility is allowed to occur overnight (20 hrs). The non-motile cells were cleaned from the top of the membrane, and the motile cells on the underside were stained and counted. **B.** The motility of T47D/Tva cells expressing vector alone (mock), FAK, SuperFAK, FAK6.7 or FRNK, were measured. The change in fold motility was plotted. Each point represents a single experiment.

Figure 6. Adhesion Independent Growth of T47D/Tva cells. A. T47D/Tva cells (3×10^4) expressing vector alone (mock) or FRNK were cultured in 0.4% agar. Eight to 10 days after the assay was setup, the number and size of the colonies was scored. (Top panel) Typical view of resulting soft agar cultures. (Bottom panel) Graphical representation comparing number of colonies formed. **B.** Lysates (25ug) of T47D expressing vector alone or FRNK after the indicated number of weeks after infection were subject to SDS-PAGE and Western blotting for FAK, to visualize FRNK expression.

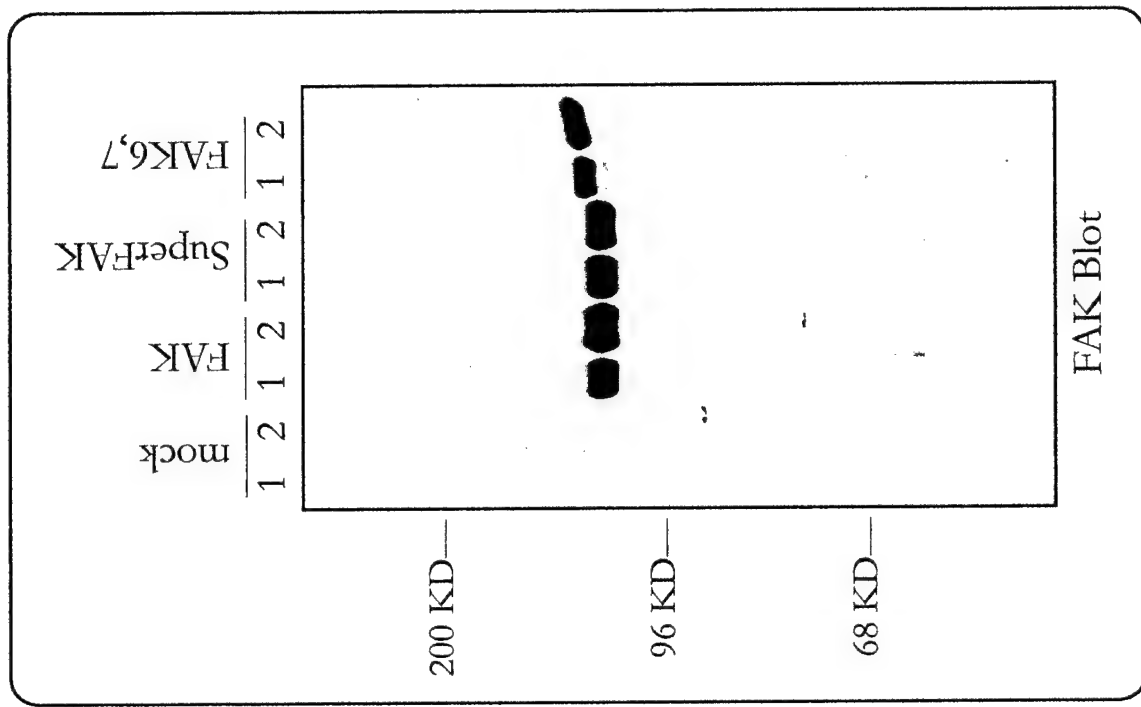
Figure 7. Cell Death Assay. T47D cells expressing vector alone or FRNK were grown for 48 hrs in suspension. The viability of the cells was measuring using a Apoptosis kit. Tunel positive cells, indicate apoptosis, and were viewed through the FITC channel. DAPI channel was used to see all the cells in the field. Control represents T47D cells treated with DNase to create positive apoptotic cells.

Figure 1

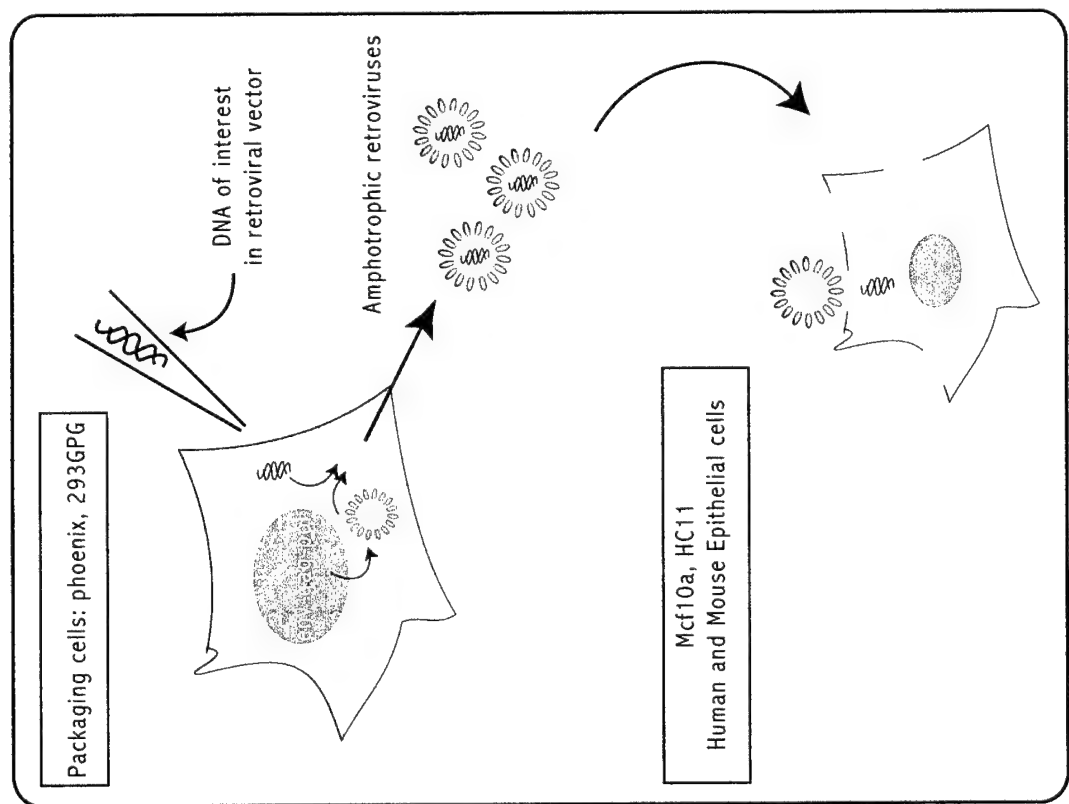
A.



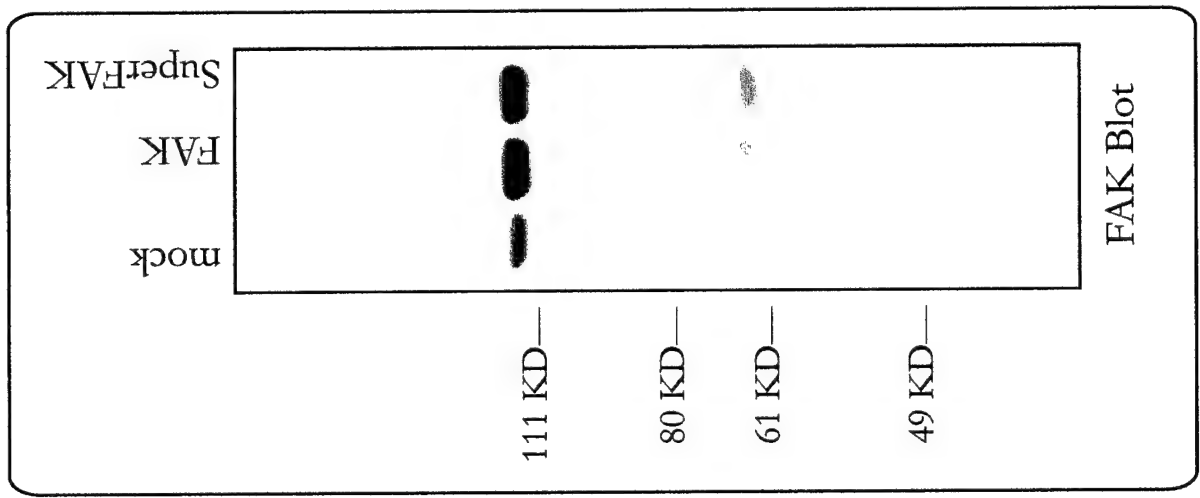
B.



A.



B.



C.

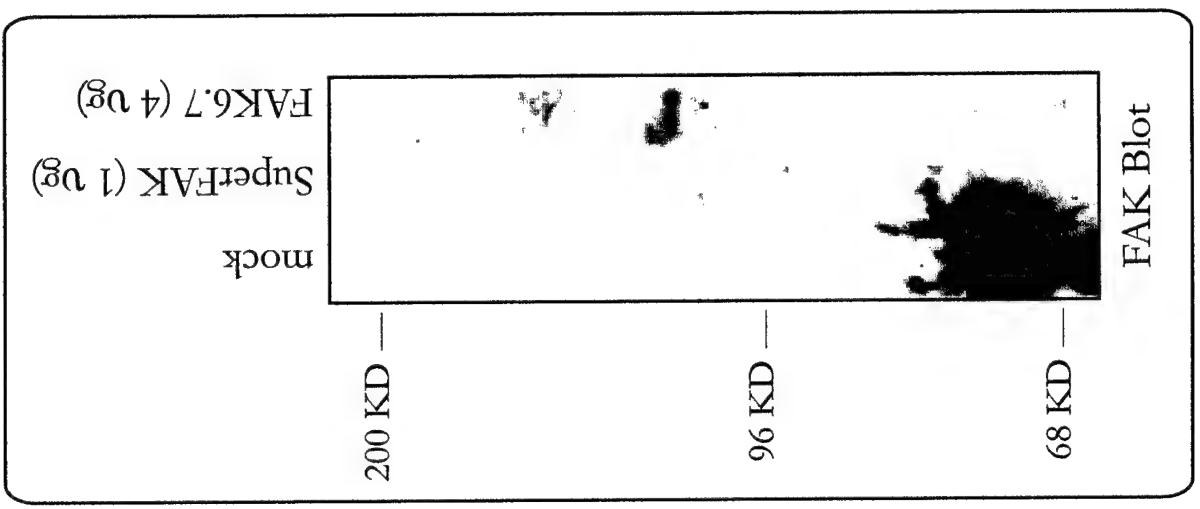


Figure 2

Figure 3

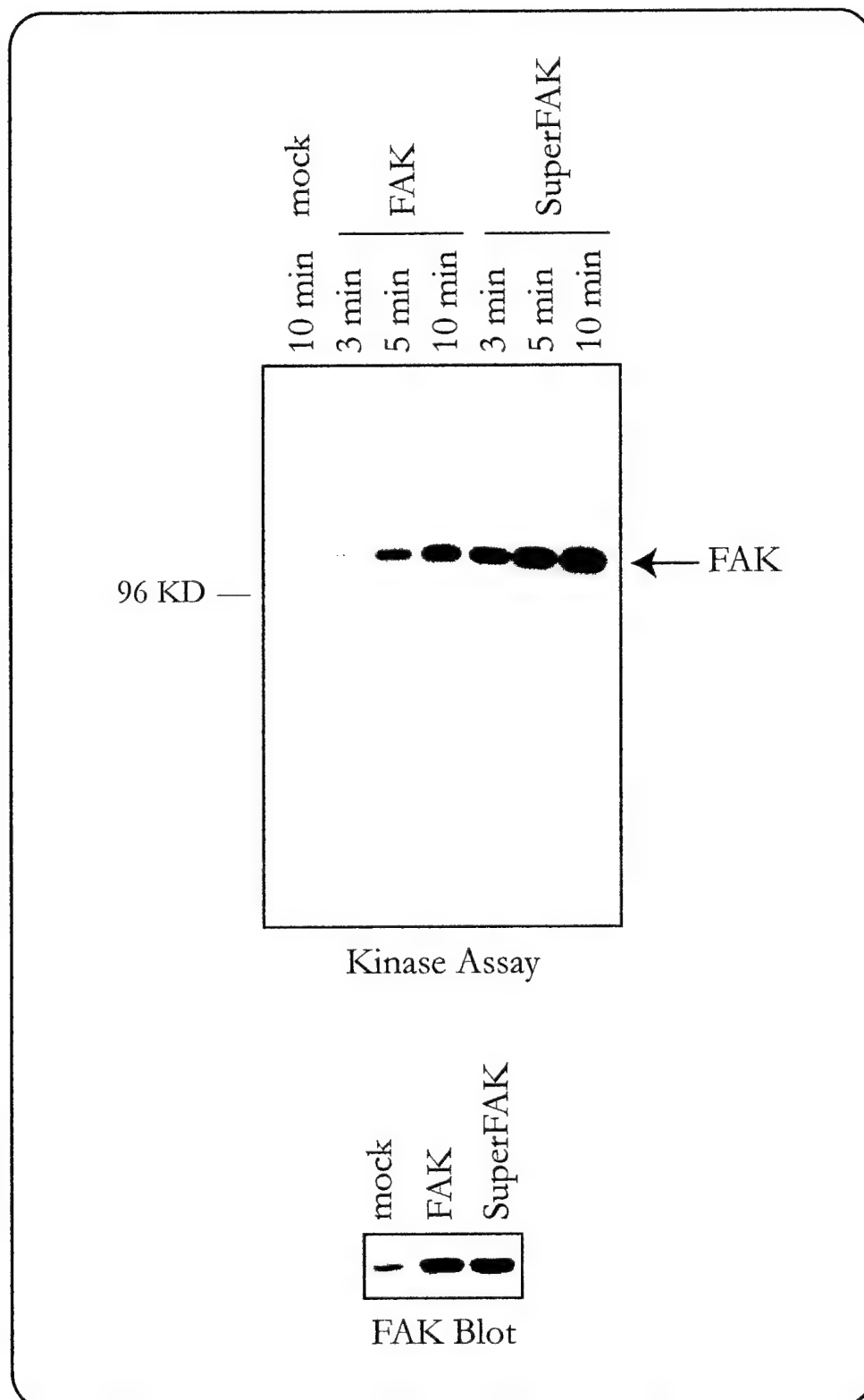


Figure 4

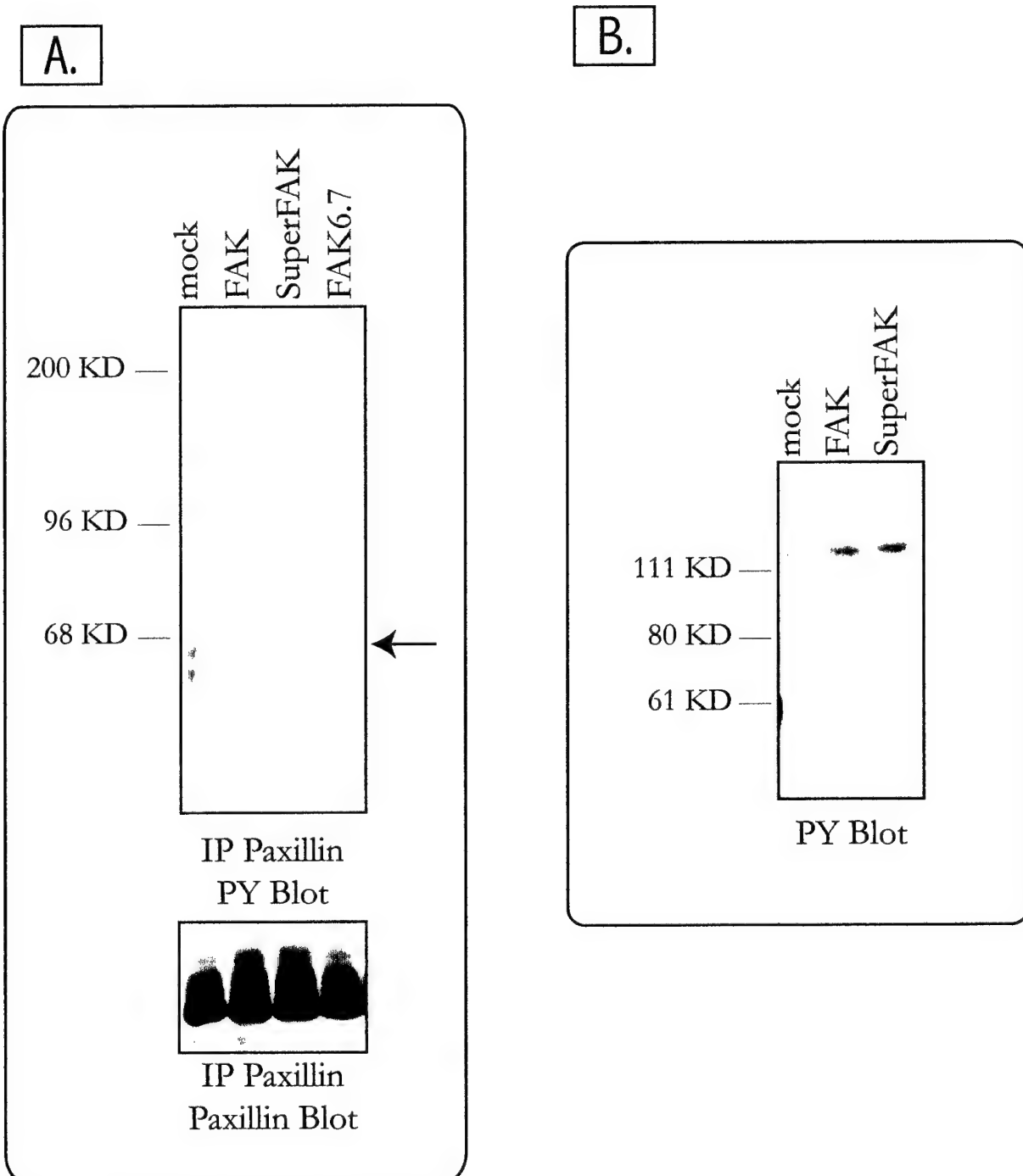
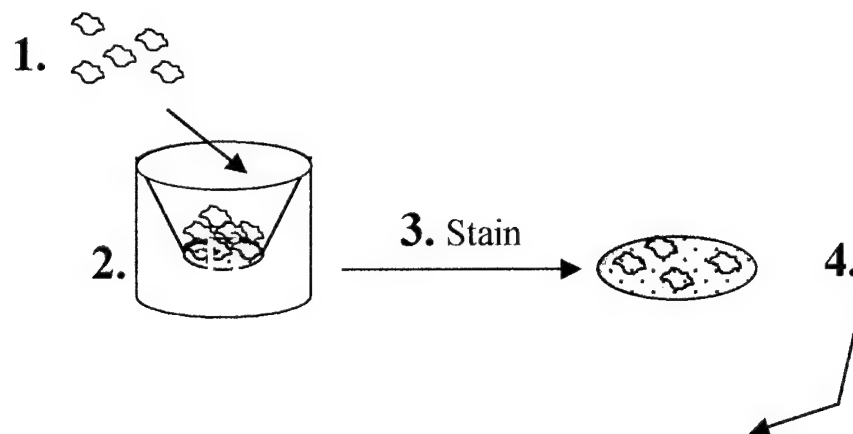


Figure 5

A.



B.

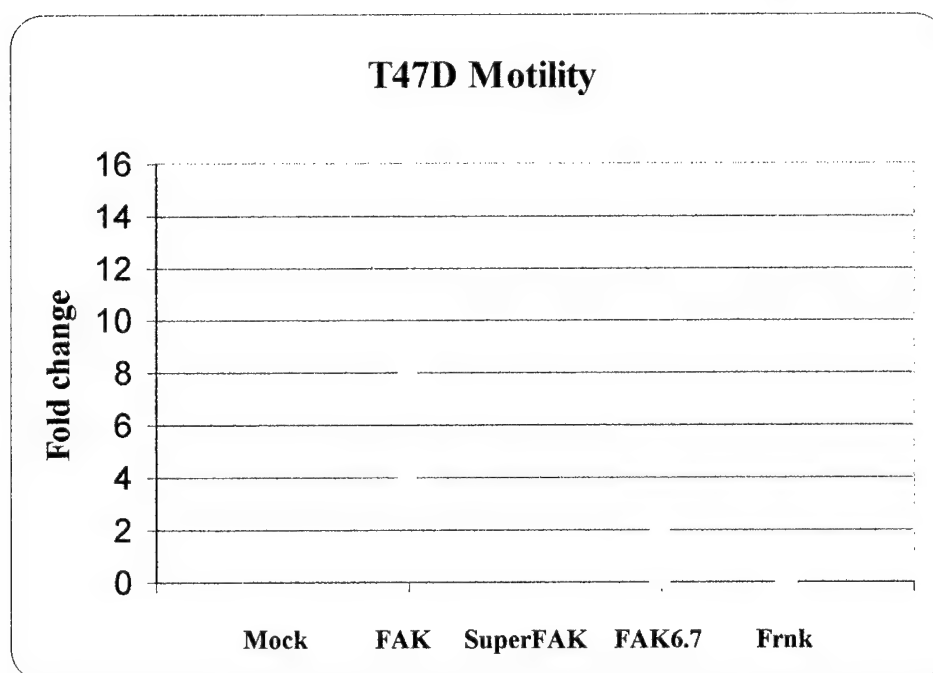
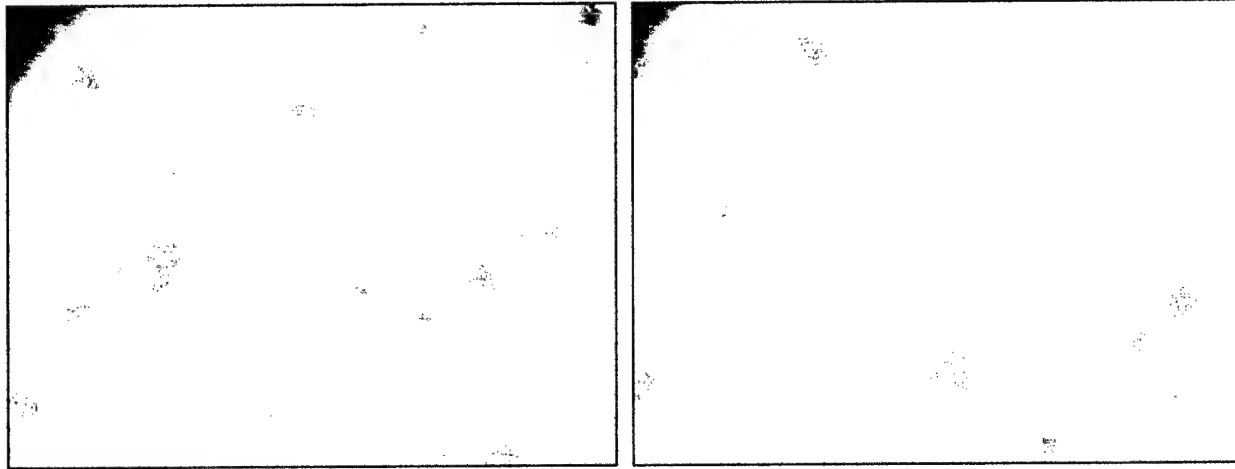


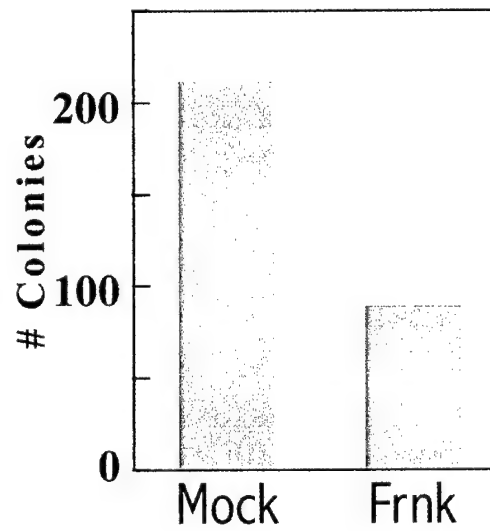
Figure 6

A.



Mock

Frnk



B.

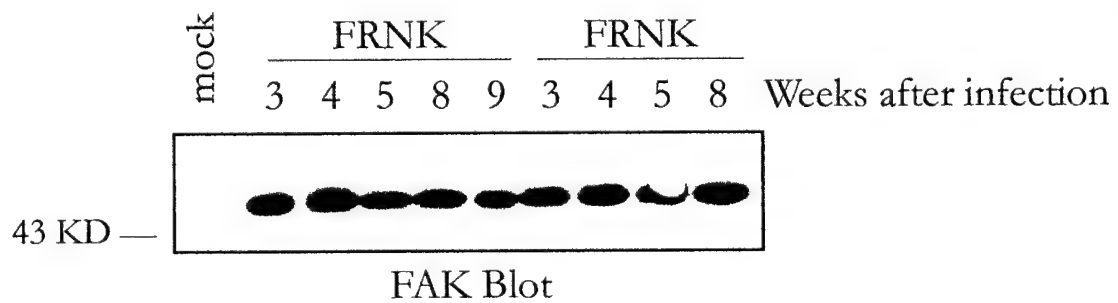
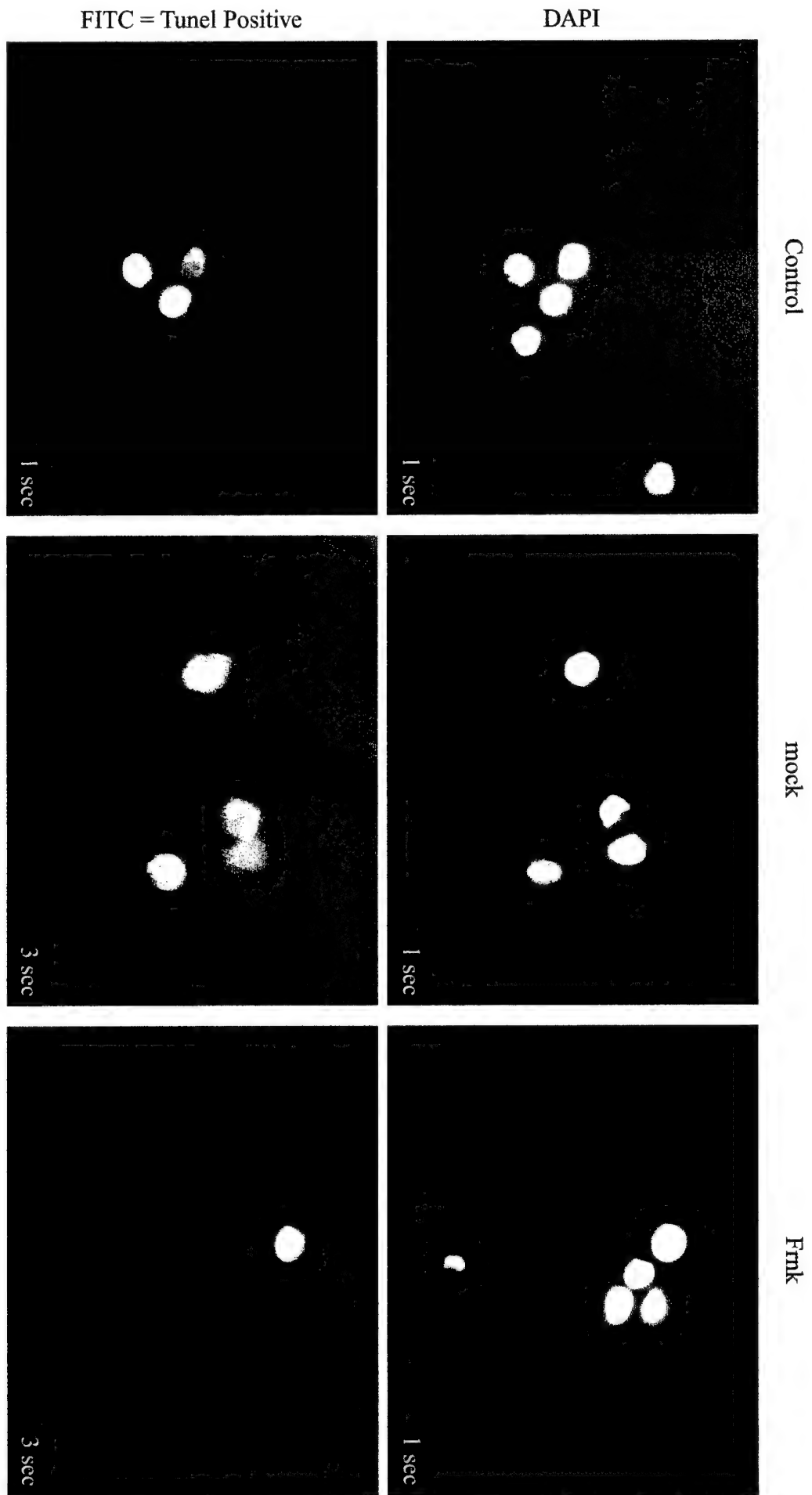


Figure 7



APPENDIX B

Manuscript in Progress: "Characterization of an activated mutant of FAK"

Characterization of an activated mutant of FAK: SuperFAK

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INTRODUCTION

The focal adhesion kinase (FAK) was first identified in Rous sarcoma virus (v-src) transformed fibroblasts (Kanner et al., 1990). The primary sequence of FAK suggested it was a non-receptor tyrosine kinase (Schaller et al., 1992). FAK localizes to focal adhesions (Schaller et al., 1992) through its focal adhesion targetting sequence (FAT) located in the carboxy terminus (Hildebrand et al., 1993). The engagement of the integrins with their extracellular matrix (ECM) ligands causes them to cluster at focal adhesions (Hynes et al., 1992; Burridge et al., 1988). The clustering of the integrins results in the phosphorylation and activation of FAK (Burridge et al., 1992; Kornberg et al., 1992; Guan et al., 1992; Hanks et al., 1992; Lipfert et al., 1992). The mechanism of activation of FAK by integrin-mediated adhesion is not clearly understood. FAK is able to associate through its amino terminal domain with $\beta 1$ integrin cytoplasmic domain (Schaller et al., 1995), and this association appears to be necessary for the adhesion-mediated phosphorylation of FAK (Burridge et al., 1992; Lewis et al., 1995). Thus, the association of FAK with integrins is thought to play an important role in the activation of FAK by facilitating the phosphorylation of FAK and the formation of signaling complexes. In addition to adhesion, treatment of cells with a variety of soluble factors, including G-protein coupled receptor agonists (Zachary et al., 1992; Kumagai et al., 1993; Seufferlein et al., 1994; Polte et al., 1994) and receptor protein tyrosine kinase ligands (Rankin et al., 1994; Abedi et al., 1995; Matsumoto et al., 1994; Kharbanda et al., 1995) can also cause FAK activation. FAK can thus be activated by a variety of factors and signaling pathways.

Upon activation, FAK autophosphorylates on tyrosine 397 (Chan et al., 1994; Schaller et al., 1994; Calalb et al., 1995; Eide et al., 1995), which is embedded in a consensus binding sequence for the SH2 domain of Src (YpAEI) (Songyang et al., 1993). Accordingly, the autophosphorylation site on FAK is able to recruit Src-like kinases, Src, Fyn, Yes, via their SH2 domains (Cobb et al., 1994; Schaller et al., 1994; Xing et al., 1994; Eide et al., 1995). In addition, FAK contains a proline rich domain upstream of Y397, which has been shown to be a consensus sequence for the SH3 domain of Src that allows for stabilization of the FAK/Src complex (Thomas et al., 1998). Once Src binds to FAK it can catalyze the phosphorylation of additional tyrosine residues on FAK, 407, 576, 577, 861, and 925 (Calalb et al., 1995; Calalb et al., 1996; Schlaepfer et al., 1994). No clear function has been found for the phosphorylation of Y407. Tyr 576, and Y577 are thought to be regulatory residues since their phosphorylation leads to maximal activation of FAK (Calalb et al., 1995). The phosphorylation of Y397, and Y925 create binding sites for SH2 domain containing proteins. The regulatory subunit of phosphatidylinositol 3' kinase (PI3K), p85, Shc, phospholipase C- $\gamma 1$ (PLC- $\gamma 1$) and Grb7, are all able to bind to phosphorylated Y397 through an SH2 mediated interaction (Chen et al., 1994; Guinebault et al., 1995; Guinebault et al., 1994; Schlaepfer et al., 1994; Bachelot et al., 1996; Zhang et al., 1999; Schlaepfer, Hunter, 1997; Zhang, Chattopadhyay, 1999; Reiske, Kao, 1999; Han, Guan, 1999). The growth factor receptor bound protein 2 (Grb2) is able to bind to phosphorylated Y925 via its SH2 domain (Schlaepfer et al., 1994; Schlaepfer et al., 1996), linking the FAK and MAPK signaling cascades (Schlaepfer, Aplin, Li).

In addition to SH2 binding sites, FAK also contains proline rich regions that serve as docking sites for the recruitment of SH3-containing proteins (Schaller et al., 1992; Thomas et al., 1998; Harte et al., 1996; Polte et al., 1995). One of the proline rich domains allows for stabilization of the FAK/Src complex (Thomas et al., 1998), as mentioned above. Two additional proline rich regions (712-723 & 874-884) are found in the carboxy terminal domain of FAK (Schaller et al., 1992; Harte et al., 1996; Polte et al., 1995). The crk-associated protein, p130cas, and the GTPase-activating protein for Rho and cdc42 (GRAF - GTPase Regulator Associated with FAK) are able to bind via their SH3 domain, to the proline rich regions in the carboxy domain of FAK (Polte et al., 1995; Harte et al., 1996; Hildebrand et al., 1996).

Sequences in the carboxy-terminus of FAK, which overlap with the focal adhesion targeting sequence, mediate the association between FAK and paxillin or talin (Turner et al., 1994; Hildebrand et al., 1995; 93? Chen et al., 1995). The overlap of the focal adhesion targeting domain sequence with the sequences responsible for the protein binding has created an interest for the potential role for paxillin and talin in mediating the localization of FAK to focal adhesions (Tachibana, Sato'1995; Cooley, Broome, 2000; Hildebrand, Schaller, 1995, Chen, Appendu'1995).

FAK has been found to be implicated in controlling a variety of integrin mediated biological processes. The increased number of focal adhesions in FAK -/- fibroblast suggests a role for FAK in focal adhesion turnover (Ilic, 1995). The introduction of FRNK, the naturally occurring dominant negative form of FAK (Schaller et al., 1993; Richardson et al., 1995), in cells inhibits their migration and spreading abilities (Romer et al., 1996; Richardson et al., 1995). Furthermore, FAK null fibroblasts showed a decreased migration ability (Ilic et al., 1995). In contrast, the expression of FAK in CHO cells has been shown to increase cell motility (Cary et al., 1996) (dependent on Y397 not kinase activity). Some of the FAK effectors thought to play a role in the FAK signal to motility include p130cas, PI3K, and most recently Shc, PLC-gamma and paxillin (Ref Guan, Guan, SHC, PLC-gamma, last two 2000 papers). The expression of FRNK or FAK peptides causes cells to arrest and die (Gilmore et al., 1996; Hungeford et al., 1996) through a process known as anoikis (loss of adhesion mediated apoptosis) (Meredith et al., 1993; Frisch et al., 1994; Re et al., 1994; Ruoslahti et al., 1994; Boudreau et al., 1995). Furthermore, a constitutively activated FAK (CD2FAK) is able to rescue cells held in suspension from undergoing anoikis (Firsch et al., 1996). PI3K has been thought to play an important role in FAK-mediated cell survival signal since this process depends on Y397 as well as the kinase activity of FAK. Interestingly, PI3K is able to mediate a survival signal through AKT (Khwaja et al.,). Recent work has also involved FAK in cell cycle regulation by mediating the G1 to S transition (Guan et Zhao et al.,).

In this paper we describe the construction and characterize the biochemical and biological capabilities of activated mutants of FAK. Although, constitutively activated FAK forms have already been described (CD2FAK, myrFAK – Chan, Aruffo'JBC1994, Igishi, Gutkind, 1999 JBC), their activation has been achieved by targeting them constitutively to the cell membrane. Since FAK has never been found to be membrane bound, the creation of an activated mutant of FAK with the appropriate cellular localization is of special interest. The activated mutants described are characterized by having elevated signaling capabilities compared to wild type FAK, including elevated catalytic activity, substrate phosphorylation and increased cell motility. The existence of

activated FAK mutants will allow for further investigation and discovery of FAK roles in cells.

METHODS

Cloning and Mutagenesis

FAK6.7 - A 6 amino acid (₃₉₃DEISGD₃₉₈) insert and a second one encoding 7 amino acids (₄₁₂KSYGIDE₄₁₈) were introduced into avian FAK cDNA by site-directed mutagenesis using the Altered Sites Kit (Promega, Madison, WI). Mutants were identified by PCR amplification and subsequently analyzed by sequence analysis using a Sequenase kit (Amersham, Piscataway, NJ). The MscI-SalI fragment (extending from amino acid 1178 in FAK into the multiple cloning sequence of the vector, downstream of the FAK stop codon) was excised from the mutagenesis vector, pALTER, and cloned into pBluescript (Stratagene, La Jolla, CA).

SuperFAK- The amino acid substitutions (K₅₇₈ K₅₈₁ → EE) were engineered into full length chicken FAK cDNA in the pBluescript vector (Stratagene, La Jolla, CA) by oligonucleotide directed PCR mutagenesis using the Stratagene Quick Change kit (Stratagene, La Jolla, CA). Mutants were identified by restriction digestion.

To verify no unintended mutations were introduced during the mutagenesis procedures, the FAK constructs were completely sequenced at the UNC-CH Automated DNA Sequencing Facility on a model 377 DNA Sequencer (Perkin-Elmer, Applied Biosystems Division) using the ABI PRISMTM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin-Elmer, Applied Biosystems Division). The full-length mutant FAK cDNA containing the appropriate mutations was subcloned into the replication competent, avian retroviral vector, RCAS (Hughes, Sutcliffe '87).

Cell Culture

Chicken embryo fibroblasts (CEF) (Willke, Tural '87) were harvested from 9-day-old embryos and grown in Dulbecco's modified Eagles's medium (Gibco/BRL, Rockville, MD) supplemented with 4% fetal bovine serum (Gibco/BRL, Rockville, MD), 1% chicken serum (Sigma, St. Louis, MO), penicillin, streptomycin, genamycin, kanamycin as described (Reynolds, Parsons '89). CEF were maintained in a 39° C and 5% CO₂ incubator.

T47D/Tva breast epithelial cells were maintained in RPMI 1640 (Gibco/BRL, Rockville, MD) supplemented with 10% fetal bovine serum (Gibco/BRL, Rockville, MD), 0.2 U/ml insulin (Gibco/BRL, Rockville, MD), penicillin, streptomycin, genamycin, kanamycin.

Transfection and Infection

CEF Transfection - CEF were transfected with RCAS DNA using LipofectAMINE PLUSTM reagent (Gibco/BRL, Rockville, MD) following the manufacturer's recommend protocol. Seven days after transfection cells were lysed and checked for expression.

Virus production - A week to 10 days after transfection, CEF cultures expressed maximal amounts of protein. At this time viral stocks were made from subconfluent cultures. The culture media was removed, 4 mls of fresh culture media was added, and the cells incubated overnight. The culture medium was collected, cells and debris were pelleted by centrifugation and virus-containing supernatants were aliquoted and stored at -70° C.

T47D Infection - Upon splitting T47D, 1 ml of virus collected from CEF expressing cells was added to the T47D cultures. Ten to fourteen days after infection, cells were lysed and checked for expression.

Preparation of Cell Lysates

Cells were lysed in modified radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl pH 7.3, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate) containing protease and phosphatase inhibitors (50 ug/ml leupeptin, 1.5 mM PMSF, 0.5% aprotinin, 1.5 mM Na Orthovanadate) for 10 min on ice¹⁶⁵. Lysates were clarified by centrifugation at 4° C. The protein concentration of the lysates was determined using the Bicinchoninic Acid Assay (BCA) kit (Pierce, Rockford, IL).

Antibodies

The polyclonal FAK antibody, BC4, and monoclonal tensin antibody, 5B9, were gifts from Dr. J.T. Parsons. The polyclonal Fyn antiserum is a generous gift from Dr. A. Veillette¹⁶⁷. The paxillin and p130cas antibodies were acquired commercially (Transduction Labs, Lexington, KY). Horseradish conjugated to protein A or anti-mouse IgG were used to recognize the primary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ). Horseradish conjugated RC20, a recombinant derivative of PY20 mAb, was used to detect phosphotyrosine levels (Transduction Labs, Lexington, KY). The polyclonal FAK Y397 phosphospecific antibody was a generous donation from Dr. E. Schaefer and is available commercially (BioSource International, Inc., Camarillo, CA).

Western blotting

After separation by SDS-PAGE, proteins were transferred onto nitrocellulose membranes. The membranes were blocked with TBS-T (10 mM Tris, 150 mM NaCl, pH 7.0, containing 0.1% Tween20) containing 5% w/v powdered milk or TBS-T containing 2% fish gelatin (Sigma, St. Louis, MO) when using the FAK Y397 phosphospecific antibody. Membranes were incubated with primary antibody in blocking solution for 1 hr at room temperature or overnight at 4° C when using FAK Y397 phosphospecific antibody. Membranes were washed 5 times for 5 minutes with TBS-T. Membranes were then incubated with appropriate HRP conjugated secondary antibody in blocking solution for 1hr. After washing with TBS-T, secondary antibody was detected using an Enhanced Chemiluminescence kit (ECL) (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunoprecipitations

Typically 0.3-1 mg of cell lysate was incubated with primary antibody on ice for 1 hr. Immunocomplexes were precipitated with protein A sepharose beads (Sigma, St. Louis, MO) for polyclonal primary antibodies, or rabbit anti-mouse IgG (Jackson Immunoresearch Laboratories Inc., West Grove, PA) pre-bound to protein A sepharose beads (Sigma, St. Louis, MO) at 4° C for 1 hr. The immunocomplexes were then washed twice with lysis buffer (modified RIPA), and twice with Tris-Buffered Saline (10 mM Tris, 150 mM NaCl, pH 7.0). Immunocomplexes were denatured and dissociated from beads by boiling in Laemmli sample buffer (Laemmli'70). The samples were then analyzed by SDS-PAGE on an 8% gel, transferred to nitrocellulose, and analyzed by Western Blotting as described above.

Kinase Assays

FAK immunocomplexes were washed twice in lysis buffer (modified RIPA) and once in kinase buffer (20 mM PIPES pH 7.4, 3mM MnCl₂ and MgCl₂). The immunocomplexes were then incubated in kinase buffer and 10 µCi γ³²P-ATP

(DupontNEN, Wilmington, DE) for the times indicated. The kinase reactions were stopped by the addition of Laemmli sample buffer (Laemmli'70) followed by boiling. The reactions were subjected to SDS-PAGE. The resulting gel was fixed in 7% acetic acid and 20% methanol for 30 minutes and dried. Autoradiography and phosphorimager analysis were used to visualize ^{32}P incorporation.

Fusion Proteins

The fusion proteins were expressed in *E. Coli* and purified as described (Smith, Johnson'88). Briefly, overnight cultures were diluted 1:10 and incubated for 2 hrs at 37° C. Expression was induced by the addition of 0.1 mM isopropyl-1-thio-B-D-galactopyranoside and further incubation at 37° C for 2 hrs. The bacteria were harvested by centrifugation. The bacterial pellet was resuspended in Sonication Buffer (1% Triton X-100 in phosphate buffered saline) containing protease inhibitors (1 mM PMSF, 0.5 mM EDTA pH 8.0, 10 µg/ml leupeptin, 10 µg/ml aprotinin). The sonication product was centrifuged to remove cellular debris. The clarified supernatants were then incubated with glutathione-agarose beads (Sigma, St. Louis, MO) for 1 h at 4° C, washed, and finally resuspended in an equal volume of phosphate-buffered saline. The fusion proteins were analyzed by SDS-PAGE and Coomassie Blue staining.

Pulldowns

In vivo association experiments were performed using glutathione-S-transferase (GST) fusion proteins containing the SH2 domains of Src (Cobb, Parsons'94) or Grb2 (a generous gift from Dr. T. Pawson). Approximately 0.5-1 mg of protein lysate was pre-cleared by incubation with GST immobilized on glutathione-Sepharose beads for 1 hr at 4° C. The cleared lysates were then incubated with 2 µg of GST alone or of GST-SH2 fusion proteins, which had been immobilized on glutathione-Sepharose beads, for 2 hr at 4° C. The beads were subsequently washed twice with lysis buffer (modified RIPA) and twice with Tris-buffered saline. The bound proteins were denatured and eluted from the beads by boiling in Laemmli sample buffer and analyzed by SDS-PAGE and Western Blotting.

Suspension and Adhesion on Fibronectin

Cells were trypsinized and washed twice in phosphate buffered saline containing 0.5 mg/ml soybean trypsin inhibitor (Sigma, St. Louis, MO). 5×10^5 cells were resuspended in plain media and kept in suspension for 45 min. Suspension cells were then collected and lysed or plated at a concentration of 2.5×10^5 cells/ml on fibronectin coated dishes (50 µg/ml) for the indicated times and finally lysed.

Motility

Motility assays were performed as described previously (Keely, Santoro'95). The underside of 12mm Transwell chambers with a 8 µm pore polycarbonate membrane (Costar, Cambridge, MA) were coated with 0.6 ml of 40 µg/ml rat tail collagen I (Collaborative Biomedical Products, Bedford, MA) for 6 hrs at 37° C. The lower chamber was washed twice and filled with plain media. T47D cells were trypsinized, counted and resuspended in medium supplemented with 5 mg/ml BSA (Sigma, St. Louis, MO) to a total concentration of 3×10^6 /ml. Half a ml of cells was added to the top chamber of the transwell. The T47D cells were allowed to move overnight (17-20 hrs) in a 37° C and 5% CO₂ incubator. At this time, non-motile cells were scraped of the top of the polycarbonate membrane. Cells that had migrated to the underside of the membrane were stained with

DiffQuick (Baxter, Miami, FL). The cells were counted across two diameters each on duplicate membranes.

RESULTS

Construction and Expression of SuperFAK and FAK6.7

SuperFAK was generated by one round of oligonucleotide site directed PCR mutagenesis through which two lysine residues in the activation loop of FAK (K578 and K581) were mutated to glutamic acids. The lysine residues are directly downstream from the two regulatory tyrosines in FAK (Y576, Y577). The idea of constructing SuperFAK was based on the K650E mutation found in constitutively activated forms of Fibroblast Growth Factor III (Weber??? See discussion) (Fig 1a). FAK6.7 was generated by introducing a 6 amino acid (KSYGIDE) and a 7 amino acid (DEISGD) long insertion starting at residue 393 and 412 respectively, which would flank the autophosphorylation site (Y397) on FAK (Fig 1). Through these insertions, the alternatively spliced FAK brain variant, described to have elevated autophosphorylation activity (Burgaya et al.), was reengineered (Fig 1a). The activated mutants of FAK, SuperFAK and FAK6.7, were cloned into the appropriate vector for expression in Chicken Embryo Fibroblasts (CEF).

In order to determine if the FAK constructs could be expressed in our CEF system, SuperFAK and FAK6.7 were transfected into CEF. Seven days after transfection the cells were lysed and the lysate (25 µg) subjected to SDS-PAGE followed by Western blotting with a polyclonal antibody for FAK (Fig 2a). The FAK constructs, SuperFAK and FAK6.7, were successfully expressed into CEF and their level of expression was comparable to the expression of wild type FAK.

SuperFAK has increased catalytic activity compared to wild type FAK

To determine the effect of the introduced mutations and insertions on the catalytic activity of the FAK constructs, SuperFAK and FAK6.7 were subjected to *in vitro* kinase assays and their catalytic activity compared to that of wild type FAK. Using a polyclonal antibody, FAK was immunoprecipitated from 600 µg-1mg of protein lysate. The immunocomplexes were washed twice in modified RIPA, and once in kinase buffer. The immunoprecipitated FAK was then incubated in kinase buffer and 10 µCi ³²P-ATP for the indicated times. The kinase reactions were stopped by the addition of Laemmli sample buffer. The reactions were subject to SDS-PAGE and autoradiography was used to visualize ³²P in the dried gels. The autophosphorylation activity of the immunoprecipitated FAK constructs was determined by monitoring the incorporation of P³² throughout time. A significant increase in the ability to autophosphorylate was observed in SuperFAK immunocomplexes (Fig 2b; top panel; lanes 4-5), and to a lesser degree FAK6.7 (Fig. 2b; top panel; lanes 6-7) compared to wild type FAK (Fig 2a; lanes 2-3). The ³²P incorporation increased with time as an indication that the assays were performed at the optimal sensitive time of the catalytic reaction. The immunocomplexes were also Western blotted with a polyclonal antibody to FAK (Fig 2b). Equivalent levels of the FAK constructs were present in the immunoprecipitates (Fig 2b) indicating that the changes in autophosphorylation were due to the different catalytic activity of the constructs and not due to different amounts of FAK protein in the reactions. Thus, the mutations and sequences introduced into SuperFAK and FAK6.7 respectively, leads to an increase in their *in vitro* kinase activity compared to wild type FAK.

Increased phosphotyrosine of downstream cellular proteins

Since SuperFAK and FAK6.7 had increased in vitro kinase activity compared to wild type FAK, the ability of these mutants to increase FAK signaling was investigated. Phosphotyrosine levels in CEF expressing wild type FAK or either one of the FAK mutants was used as a measure of FAK signaling. Whole cell lysates (25 μ g) from CEFs expressing wild type FAK or either one of the FAK mutants were subject to SDS-PAGE and Western blotted with a phosphotyrosine specific antibody (Fig 3a). An increase in phosphotyrosine content of cellular protein pool was observed in SuperFAK overexpressors, and to a lesser degree FAK6.7, compared to lysates of CEF overexpressing wild type FAK. The phosphotyrosine signals seem to concentrate around proteins of 200, about 100 and 68 KDa in size (Fig 3a, arrows). FAK is known to be a 125 KDa protein (Schaller'92, Hanks'92), furthermore known substrates of FAK, tensin (MCB/MBC mike) and paxillin (turner, mike), are 200 and 68 Kda respectively.

In order to identify the proteins that were being hyperphosphorylated, tensin, FAK, and paxillin were immunoprecipitated from 500 μ g-1 mg lysate from CEF overexpressing wild type FAK or either one of the FAK constructs. An additional substrate for FAK phosphorylation, p130cas (Polte), was also immunoprecipitated in order to visualize its level of tyrosine phosphorylation. The immunocomplexes were washed, dissociated and separated by SDS-PAGE followed by phosphotyrosine Western blotting (Fig 3b-e; top panels). An elevation in the amount of phosphotyrosine was observed in tensin (Fig 3b; top panel), FAK somewhat (Fig 3c; top panel) and especially paxillin (Fig 3d; top panel) immunocomplexes from cells expressing SuperFAK, and a lesser increase in phosphotyrosine was observed in FAK6.7 expressing cells. In contrast, no significant change in the phosphotyrosine content of p130cas was observed (Fig 3 e; top panel). As a control, the immunocomplexes were Western blotted for the protein being immunoprecipitated (Fig3b-e; bottom panels), tensin, FAK, paxillin and p130cas. The equal amount of protein being immunoprecipitated in each case indicates that the increase in phosphotyrosine is not due to high levels of the protein being analyzed but to a real change in their phosphorylation content. These observations indicate that the activated FAK constructs, SuperFAK, and to a lesser degree FAK6.7, are able to increase the FAK phosphorylation signals as measured by the phosphorylation of FAK substrates. Furthermore, the level of phosphorylation of the FAK substrates seems to correlate with the catalytic activity of the construct since SuperFAK had a greater effect on phosphorylation than FAK6.7, and also showed higher activity in vitro (Fig 2b). The same was true between SuperFAK and FAK, and FAK6.7 and wild type FAK. Thus, by increasing the catalytic activity of FAK, we have been able to increase downstream phosphorylation signals.

SuperFAK and FAK6.7 Signals are regulated by Adhesion but the Kinase Activity is Maintained

Cell adhesion is known to be the activation signal for FAK-mediated signal transduction. When cells loose their adhesion, FAK gets dephosphorylated, and its catalytic activity and its downstream signals are turned off (Hanks'92). The regulation or dependence of the FAK mutants on adhesion for downstream signaling was investigated. For this purpose, the cellular phosphotyrosine content was monitored in CEF expressing wild type FAK or either one of the FAK mutants from cells that were either kept in culture or held in suspension for 45 min. Whole cell lysates (25 μ g) of cells in culture or in suspension, were subjected to SDS-PAGE followed by Western blotting with a

phosphotyrosine antibody (Fig 4a). Again we observe the ability of SuperFAK, and to a lesser degree FAK6.7, to elevate the cellular phosphotyrosine pool to higher levels than wild type FAK (Fig 4a; Lanes 3,5,7). When the adhesion signal was taken away, in every case, the cellular phosphotyrosine was completely lost. These observations indicate that the downstream phosphorylation signals mediated by SuperFAK and FAK6.7 are adhesion dependent just like wild type FAK.

Kinase activity is not lost in suspended cells

When cells lose their adhesion signal and FAK is dephosphorylated, its kinase activity is turned off ultimately leading to a cessation in downstream signals. *In vitro* kinase assays were performed in order to investigate if the decreased downstream phosphorylation signals observed upon loss of adhesion in SuperFAK and FAK6.7 overexpressors was due to a loss in catalytic activity. FAK or either one of the activated FAK mutants was immunoprecipitated from cultured CEFs or cells held in suspension. The immunocomplexes were washed as usual followed by a wash in kinase buffer. The immunoprecipitated FAK constructs were then incubated in kinase buffer with ^{32}P for 5 min. The kinase reactions were stopped through the addition of Laemmli sample buffer. The samples were then subject to SDS-PAGE, and the incorporation of ^{32}P was visualized through autoradiography. Again, the autophosphorylation activity of SuperFAK, and to a lesser degree FAK6.7, was increased compared to wild type FAK (Fig 4b; top panel; lanes 3,5,7), which agrees with previous observations (Fig 2b). The kinase activity of SuperFAK and FAK6.7 as well as FAK decreased in the absence of an adhesion signal (Fig 4b; top panel; compare lanes 3 & 4, 5 & 6, 7 & 8). However, SuperFAK and FAK6.7 still exhibited significantly higher catalytic activity compared to wild type FAK (Fig 4b; top panels; compare lanes 4,6,8), which was especially robust in the case of SuperFAK. These observations might explain why the phosphorylation at the level of FAK was not completely lost in the absence of an adhesion signal (Fig 4a). Furthermore, these results indicate that SuperFAK, and to a lesser degree FAK6.7, are better kinases than their wild type counterpart in the presence or in the absence of adhesion.

Rescue of FAK signal upon Adhesion To Fibronectin

The catalytic activity of FAK can be turned on or rescued when cells that have lost their adhesion, upon re-adhesion on an extracellular matrix (hanks'92). In order to test whether the activated FAK mutants could respond to this type of physiological signal, cells that had been kept in suspension were allowed to attach onto fibronectin. The phosphorylation of paxillin was used as a measure of FAK activity. Paxillin was immunoprecipitated from 0.5 μg - 1 mg of lysates of cultured cells, cells kept in suspension for 45 min, or cells replated on fibronectin-coated dishes after being held in suspension. The paxillin immunocomplexes were then Western blotted with a phosphotyrosine antibody or a paxillin, the latter demonstrates that equal amounts of protein are being immunoprecipitated (Fig 5; bottom panel) and thus the phosphotyrosine changes are true changes and not due to changes in the amount paxillin being immunoprecipitated. Upon underexposure of the blot, SuperFAK, and to a lesser degree FAK6.7 are able to further increase the level of tyrosine phosphorylation on paxillin compared to wild type protein (data not shown and Fig 5; top panel; lanes 6, 10, 14), which agrees with previous observations (Fig 3d) and demonstrates the increased ability of the FAK constructs to send downstream FAK signaling. Upon loss of adhesion, the

paxillin phosphorylation is lost no matter what FAK construct is being expressed (Fig 5; top panel; compare Cul vs Su), which correlates with previous observations (Fig 4a), and demonstrates the necessity of an adhesion signal for successful FAK signaling. Furthermore, fibronectin-mediated adhesion rescued FAK signals as indicated by the phosphorylation of paxillin in all the cells (Fig 5; top panel; FN lanes). The level of paxillin phosphorylation was significantly higher in cells expressing SuperFAK or FAK6.7, compared to CEF expressing wild type FAK (Fig 5; top panel; lanes 8 & 9 vs 12 & 13, and 16 & 17). These observations not only confirmed that SuperFAK and FAK6.7 can elevate downstream FAK signals, but also demonstrate that the effects of the activated FAK mutants, occur upon physiologically relevant stimulation.

Phosphorylation Status of Tyrosine Residues in SuperFAK and Fak6.7

Autophosphorylation Levels and Src Binding

One possible mechanism through which the activated mutants might be able to elevate FAK catalytic activity and downstream signaling, could be through an increase in autophosphorylation, which in turn would increase Src binding to the FAK constructs. In order to investigate the level of phosphorylation on Tyr397, and the formation of the Src-FAK complex, three different approaches were taken. Lysates (25 μ g) of CEF expressing wild type FAK, SuperFAK or FAK6.7 were subjected to SDS-PAGE and Western blotted with a phospho-specific antibody against Y397 (α -PY397). To control for specificity, a FAK mutant in which Y397 had been mutated to phenylalanine (F), and thus is unable to be phosphorylated at this site, was used as a negative control (Fig 6a; lane 3). The CEF expressed equal amounts of protein as indicated by a FAK Western blot of the same lysates (25 μ g) (Fig 6a; bottom panel), indicating that equal amounts of protein were being analyzed. No changes in phosphorylation levels of Y397 were observed between wild type FAK, SuperFAK and FAK6.7 (Fig 6a; top panel), indicating that the neither SuperFAK nor FAK6.7 had gained increased phosphorylation at Y397 *in vivo*.

The phosphorylation of Y397 on FAK creates a binding site for the SH2 domain of Src (Cobb, Schaller). To analyze the ability of the activated FAK mutants to recruit Src, and as a measure of the level of autophosphorylation, 2 μ g of a GST fusion protein containing the SH2 domain of Src was used to pulldown FAK and the FAK constructs out of 0.5 μ g-1 mg of CEF lysates that had been pre-cleared with 20 μ g of GST. The GST-SrcSH2 pulldowns were washed and then boiled in Laemmli sample buffer and the samples were western blotted for FAK to analyze the amount of FAK or FAK construct associating with the SrcSH2. As a control, GST was used in a pulldown to ensure that non-specific FAK association was occurring (Fig 6b; lane 1). No significant difference was found in the amounts of exogenous wild type FAK or activated FAK mutants being pulled down by GST-SrcSH2 (Fig 6b; lane 3-5). These observations demonstrate that there are no changes in the capability of neither SuperFAK nor FAK6.7 to associate with Src compared to wild type FAK. Furthermore, since the autophosphorylated Y397 is the site of Src binding, these observations also suggest that there are no changes in the levels of autophosphorylation of the activated FAK mutants, in agreement with the Y397 phosphospecific Western blot (Fig 6a).

Co-immunoprecipitations were also performed to analyze the association of fyn, a Src-like kinase, and FAK. Fyn was immunoprecipitated from 0.5 μ g-1 mg of CEF lysates that overexpressed wild type FAK, SuperFAK or FAK6.7. The immunocomplexes were washed, subject to SDS-APGE and Western blotted for FAK or Fyn (Fig 6c). If the levels

of autophosphorylation were altered in any of the activated FAK mutants, a corresponding change in amount of FAK associating with Fyn, which binds to the phosphorylated Y397 residue, would have been expected. Instead, no significant changes in the amount of exogenous FAK associating with Fyn was observed (Fig 6c; top panel; lanes 3-5). As a control, secondary antibody was used alone in a immunoprecipitation to demonstrate that the Fyn association with FAK was specific (Fig 6c; top panel; lane 1). In addition, the immunoprecipitates were also Western blotted with Fyn to make sure that equal amount of Fyn were being analyzed (Fig 6c; bottom panel). These observations indicate that SuperFAK and FAK6.7 and wild type FAK associate with the same amount of Fyn, thus suggesting that SuperFAK and FAK6.7 have the same level of autophosphorylation as wild type FAK.

The observations obtained through the use of a Y397 phosphospecific antibody, GST-SrcSH2 pulldowns, and Fyn-FAK co-immunoprecipitations indicate that the mutations and insertions introduced in SuperFAK and FAK6.7 respectively, have no effect in the level of phosphorylation of Y397, nor their ability to associate with Src-like kinases.

Altered Phosphorylation Levels of Tyr925

A possible mechanism leading to increases in downstream signaling of SuperFAK and FAK6.7 could be related to an increase in tyrosine phosphorylation, which in turn could potentially increase the recruitment of SH2 containing proteins, some of which mediate FAK signals. The phosphorylation of Y925 in the carboxy-terminus of FAK has been shown to create a binding site for the SH2 domain of Grb2 (Schlaepfer). In order to analyze the ability of SuperFAK and FAK6.7 to recruit Grb2, a GST fusion protein of the Grb2-SH2 was used to pulldown FAK out of CEF lysates. The GST-Grb2SH2 pulldowns were then analyzed by western blotting for FAK. Interestingly, higher levels of SuperFAK were pulldown compared to wild type FAK. In contrast, no FAK6.7 was observed in the pulldowns. The increased ability of SuperFAK to recruit Grb2 suggests that compared to wild type FAK, SuperFAK has higher levels of phosphorylation of Y925. FAK6.7, however, was not found associated with Grb2 indicating a potential loss in Y925 phosphorylation compared to its wild type counterpart.

Increased Motility of T47D cells expressing SuperFAK

Since SuperFAK, and to a lesser degree FAK6.7, were able to increase FAK signaling at the biochemical level, their ability to increase biological processes mediated by FAK were tested. FAK, SuperFAK and FAK6.7 were expressed in T47D cells, a breast cancer epithelial cell line. Using a transwell system, the haptotactic motility ability of T47D was observed to increase in FAK overexpressors, which is an expected result based on the known role of FAK in mediating a motility signals (romer, guan, etc). SuperFAK was able to increase the motility of T47D to higher levels than T47D cells expressing FAK. Interestingly, FAK6.7 was not only unable to increase the haptotactic response of T47D cells, but it seemed to inhibit their motility down to mock levels. These observations demonstrate the ability of SuperFAK to increase a FAK-mediated biological response and the effect of FAK6.7 on motility, together with its biochemical effects, create a new set of intriguing questions about the mechanism through which FAK mediates motility.

DISCUSSION

In this study, we have been able to create activated mutants of FAK, SuperFAK and FAK6.7. SuperFAK, and to a lesser degree, FAK6.7, showed and increased in their *in vitro* catalytic activity compared to wild type FAK. The change in activity translates itself into increased downstream biochemical signals, as represented by the phosphorylation of FAK effectors, paxillin and tensin. Furthermore, the elevation of the FAK signal by expressing SuperFAK ultimately leads to an increased motile phenotype in cells. Thus, SuperFAK is an additional powerful tool that can be used to study the consequences of increased and/or aberrant FAK signaling in a variety of physiological contexts.

The substitution of the double lysine (##) by glutamic acids in the context of FAK has the same effects on FAK as the original mutation had in FGFR (Weber), which includes increased kinase activity and downstream biochemical and cellular events mediated by the kinase. Although the structure of FAK has not yet been described, its central kinase domain has a high sequence identity with tyrosine kinases (Schaller 1992). Furthermore, the study of a variety of tyrosine kinase structures has given us a better understanding of their mechanism of activation (Reviews). It is thought that phosphorylation of tyrosine residues in the kinase domain, probably Y576 and Y577 in the case of FAK, causes the activation loop to undergo a conformational change. The negative charge introduced by the phosphate groups leads to a repulsion between the activation loop and other key residues in the ATP binding pocket of the kinase domain. This conformational change allows the activation loop to fold back out, making the ATP binding site accessible. In the case of SuperFAK, the negatively charged glutamic acid residues are thought to mimic the conformational change that must occur when the regulatory phosphotyrosines (576/577) in the activation loop of the kinase get phosphorylated. Thus, causing SuperFAK to be in an open or active conformation at all times.

On the other hand, FAK6.7 has been described as an alternatively spliced brain variant of FAK (Burgaya). The presence of two additional exons, 6 and 7 amino acid residues in length, flanking the Y397 of FAK, leads to an increase in the autophosphorylation ability of the kinase and thus it was thought that these changes might increase the ability of FAK to send downstream biochemical and biological signals, maybe via increased Src recruitment. We did observe an increase in the *in vitro* kinase activity of FAK6.7 and phosphorylation of downstream substrates of FAK, paxillin and tensin, however the motility of the cells seemed to be inhibited. One explanation for the contradictory observations could be that the insertion of the residues around the autophosphorylation tyrosine (Y397), also disturbed the association of FAK6.7 with known binding partners of FAK. PI3K, PLC γ , Grb7, and Shc are known to bind to the phosphorylated Y397 (REF). Furthermore, PI3K, PLC γ , Shc, and Grb7 have all been implicated in mediating migration responses, and more specifically PI3K is known to be involved in FAK-mediated motility (REF). Thus, if FAK6.7 has lost its ability to bind to some or any of these partners, it might explain the decrease or inhibition of the motility response.

One point worth discussing refers to the regulation of SuperFAK and FAK6.7. It was interesting to observe that although the kinase activity of SuperFAK and FAK6.7 decreased when the cells did not receive an adhesion signal, SuperFAK and FAK6.7 still showed a significantly higher kinase activity compared to wild type FAK. However, no

phosphorylation of downstream effectors was observed in the absence of an adhesion signal in cells expressing either one of the activated mutants. In addition, the level of association between SuperFAK and FAK6.7 and the FAK substrates that we looked at when cells were kept in suspension was comparable to that of wild type FAK (data not shown). These observations indicate that no matter how catalytically active SuperFAK and FAK6.7 are they must localize to the appropriate subcellular space in order to send and affect FAK downstream signals. Thus, we can conclude that correct cellular localization might be a critical aspect of successful FAK signaling.

How is the activation of SuperFAK and FAK6.7 occurring? The molecular mechanism of activation of the FAK signal described up to date demonstrate that FAK autophosphorylates upon localization to focal plaques, creating a binding site for the recruitment SH2-containing proteins, including Src-like kinases (Cobb, Schaller, REF). Src can then further phosphorylate FAK on its regulatory tyrosine residues (Y576,577) leading to maximal activation of the kinase (REF). Thus, one of the effects of introducing the mutations and insertions into SuperFAK and FAK6.7 respectively might be to increase their ability to autophosphorylate thus allowing for further Src recruitment. However, Western blotting of the immunoprecipitated FAK constructs with the phosphospecific antibody to the autophosphorylated tyrosine on FAK (Y397) indicated that there are no significant changes in the levels of autophosphorylation phosphorylation between the activated FAK mutants and wild type FAK. Burgaya et al., showed recently, through the use of a different phosphospecific antibody to the phosphorylated Y397 on FAK, that the neuronal FAK variant, of which FAK6.7 is a reconstruction, has increased levels of autophosphorylation (Burgaya et al., 2000). One of the possible reasons for the discrepancy might be that the antibody used in our studies is unable to bind to FAK6.7. The inserts flanking Y397 might have disrupted the epitope recognized by the antibody, or may have caused a conformational change that disrupts the antibody from binding to FAK6.7. In addition to the phosphospecific antibody studies, pulldowns and immunoprecipitations demonstrated that the association of SuperFAK and FAK6.7 with Src were comparable to the association between Src and wild type FAK. These results concur with the previous observations, suggesting that the autophosphorylation levels on neither SuperFAK nor FAK6.7 remain unchanged. But most interesting of all, these observations are suggesting that the effects of the SuperFAK and FAK6.7 on downstream signaling might be occurring independently of Src. Such possibility is further reinforced by the change in substrate specificity that was observed when the phosphorylation of FAK effector proteins was analyzed. Although paxillin and tensin phosphorylation was elevated in cells expressing SuperFAK and FAK6.7, p130cas phosphorylation remained unchanged. Interestingly, p130cas is thought to be a better substrate for Src than FAK (Vuori, Ruoslahti, 1996). In Src $-/-$, Fyn $-/-$, and Yes $-/-$ cells, p130cas appears to be the most hypophosphorylated protein (Bockholt, Burrige, 1995; Vuori, Ruoslahti, 1996). In addition, Vuori et al. showed that Csk $-/-$ cells, Csk being a negative regulator of Src (Thomas, Brugge, 1997), leads to the hyperphosphorylation of p130cas and in contrast p130cas phosphorylation is not compromised in FAK $-/-$ cells. Furthermore, the phosphorylation of p130cas by FAK is dependent on Y397, the binding site for Src. These observations suggested that Src is a better kinase for p130cas phosphorylation and that FAK is able to phosphorylate p130cas by recruiting Src into a complex via its autophosphorylation site (Y397). In our studies, we showed that SuperFAK and FAK6.7

recruit the same amount of Src as does wild type FAK, which correlates with their steady levels of phosphorylation on Y397. Furthermore, we show that SuperFAK, and to a lesser degree FAK6.7, are able to increase the phosphorylation levels of FAK substrates, tensin and paxillin, but not p130cas. These observations suggest that the activated mutants described herein are not having any effect on Src mediated signals, instead the biochemical and biological changes observed upon expression of SuperFAK and FAK6.7 seem to be a direct product of increased FAK signaling. A FAK-dependent mechanism of Src activation has been proposed (Thomas, Ellis'98; Alexandropoulos'96; Schlaepfer, Jones'98 CHECK/GET), thus it could be feasible that the effects of the activated FAK mutants might occur through activation of Src.

One interesting observations in this study was the differences associated with Y925 between the activated FAK mutants. Compared to wild type FAK, SuperFAK demonstrated an elevated association with Grb2, suggesting an increased level of Y925 phosphorylation on SuperFAK, the binding site for Grb2. However, if there are no changes in the association between Src and SuperFAK, and Src is responsible for phosphorylation of Y925, how is SuperFAK increasing its Y925 phosphorylation. Again, SuperFAK might be affecting Src activation. On the other hand, phosphatases have been suggested to negatively regulate FAK activity, including Shp2 and PTEN (Manes, Mira'99; Miao, Burnett'00; Oh, Gu'99; Fujioka, Matozaki'96, Tsuda, Matozaki'98; Yu, Qu'98; Oh, Gu'99---Tamura, Gu'98'99'99). SuperFAK could be turning off a Y925 specific phosphatase by phosphorylating it, ultimately leading to conserved phosphorylation on Y925 in SuperFAK compared to wild type FAK. One intriguing possibility, although it remains to be demonstrated, is that through a change in specificity or due to the level of kinase activity, SuperFAK has acquired the ability to phosphorylate Y925 directly. FAK6.7, in contrast to SuperFAK, seems to have lost the ability to bind Grb2, as shown through pulldowns, suggesting a loss Y925 phosphorylation. It is not due to a loss in Src binding as the association between FAK6.7 and Src, and FAK6.7 Y397 phosphorylation remained the same as in wild type FAK. But could FAK6.7 be inhibiting Src activity, and thus blocking Y925 phosphorylation. In this scenario, Src would not be able to activate FAK6.7 and thus the construct would not be active, which is not the case. Is FAK6.7 having an effect on a phosphatase, activating it, and leading to complete loss of Y925 phosphorylation? The question still remains. However, the observation that Y925 and thus Grb2 binding might be blocked in FAK6.7 might explain the loss of motility of cells expressing FAK6.7. It has been suggested that FAK might be involved in Hepatocyte Growth Factor/Scator Factor motility in MDCK via its binding to Grb2 (Lai, Kao, 2000). However, the effects of FAK6.7 on motility were on a haptotactic mediated response, thus this possibility might not accurately explain the effects of FAK6.7 in our studies.

Figure 1

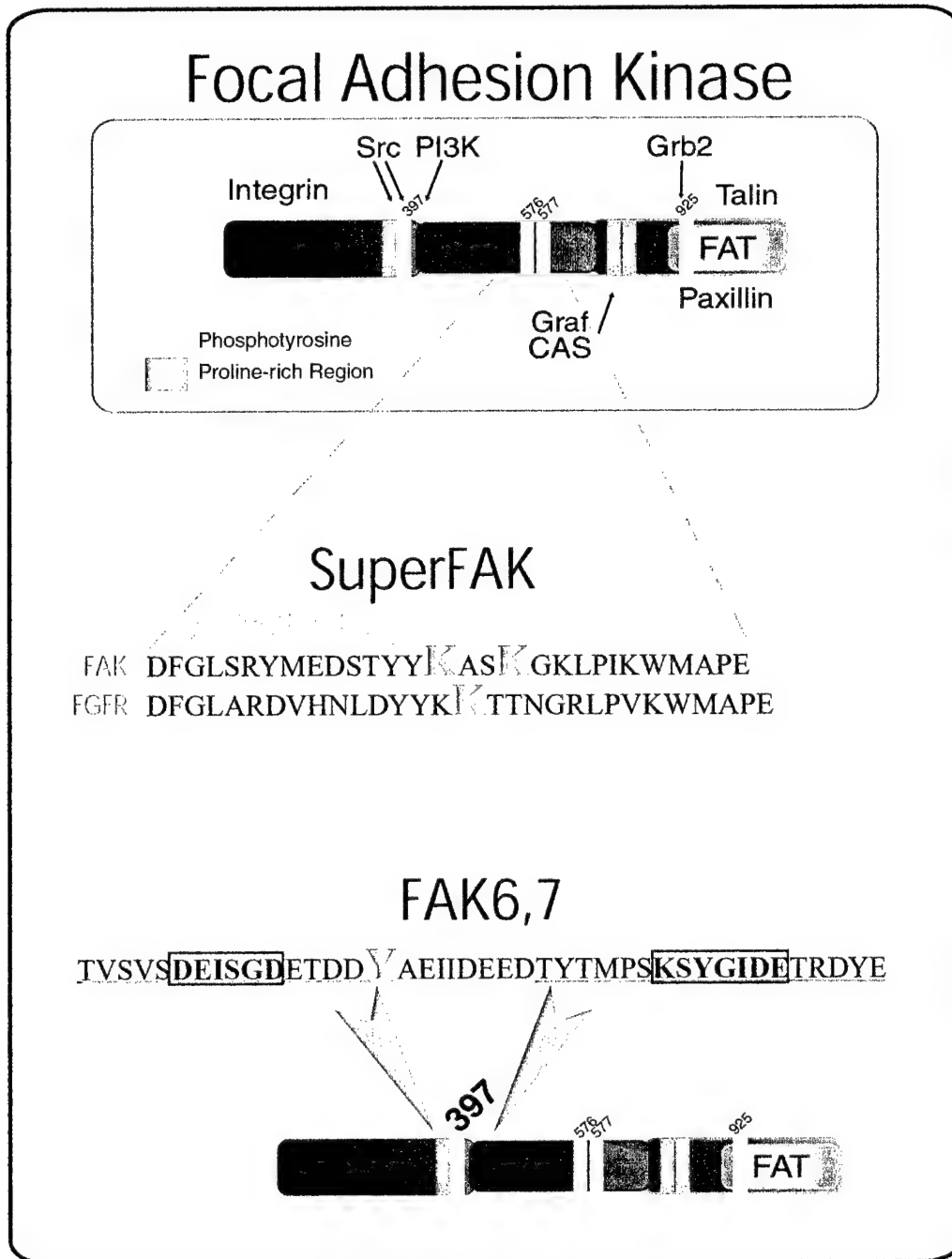
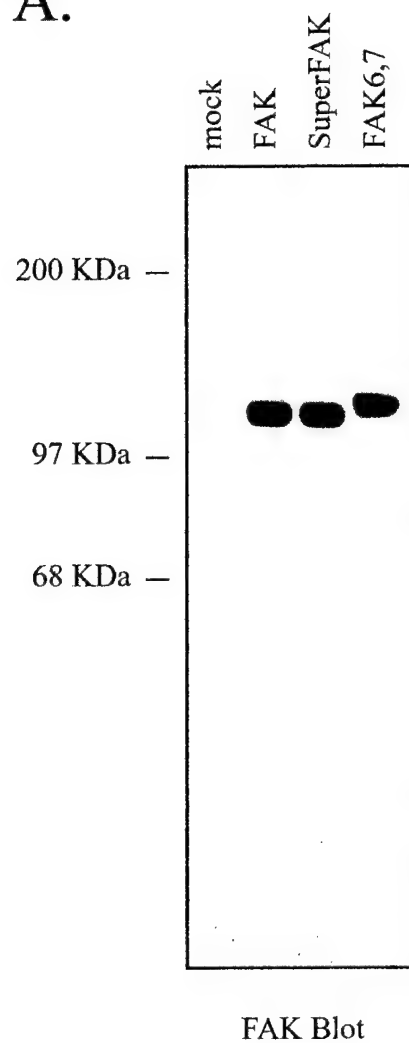


Figure 2

A.



B.

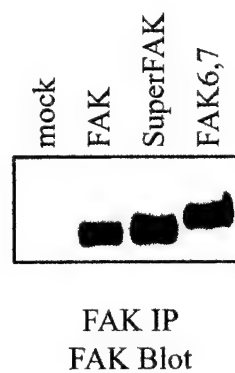
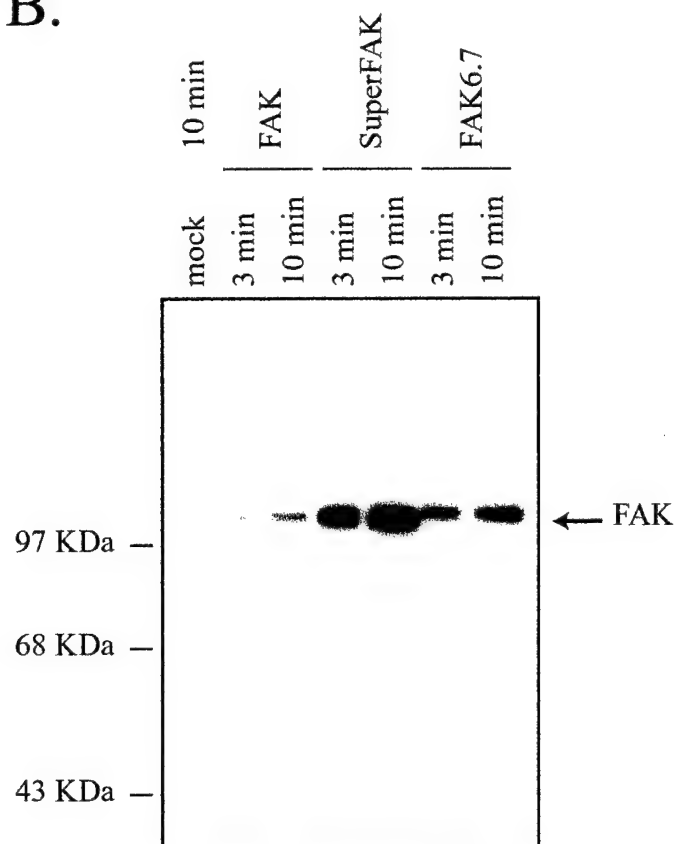


Figure 3

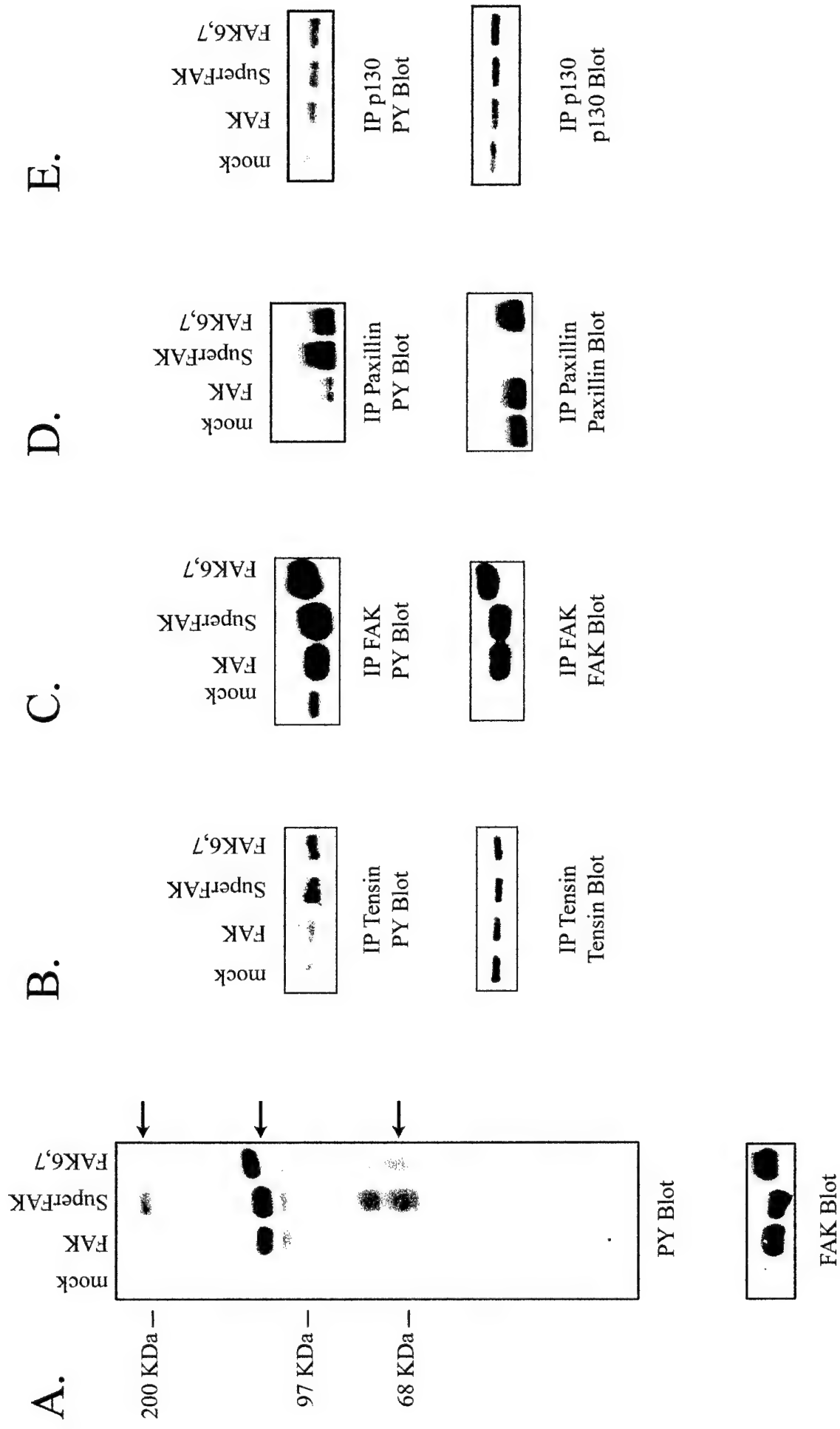
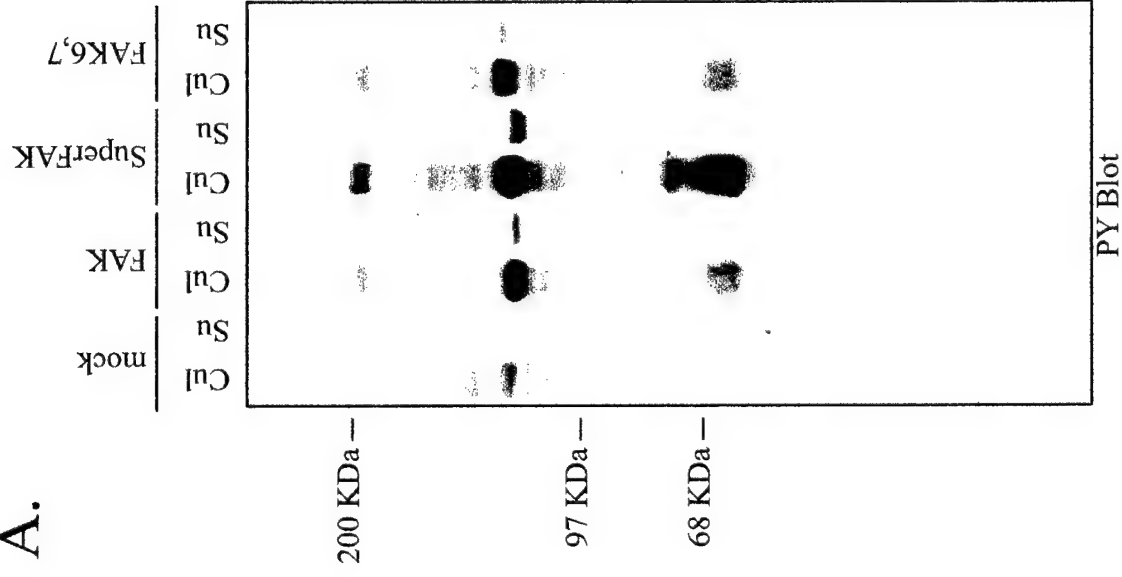


Figure 4

A.



B.

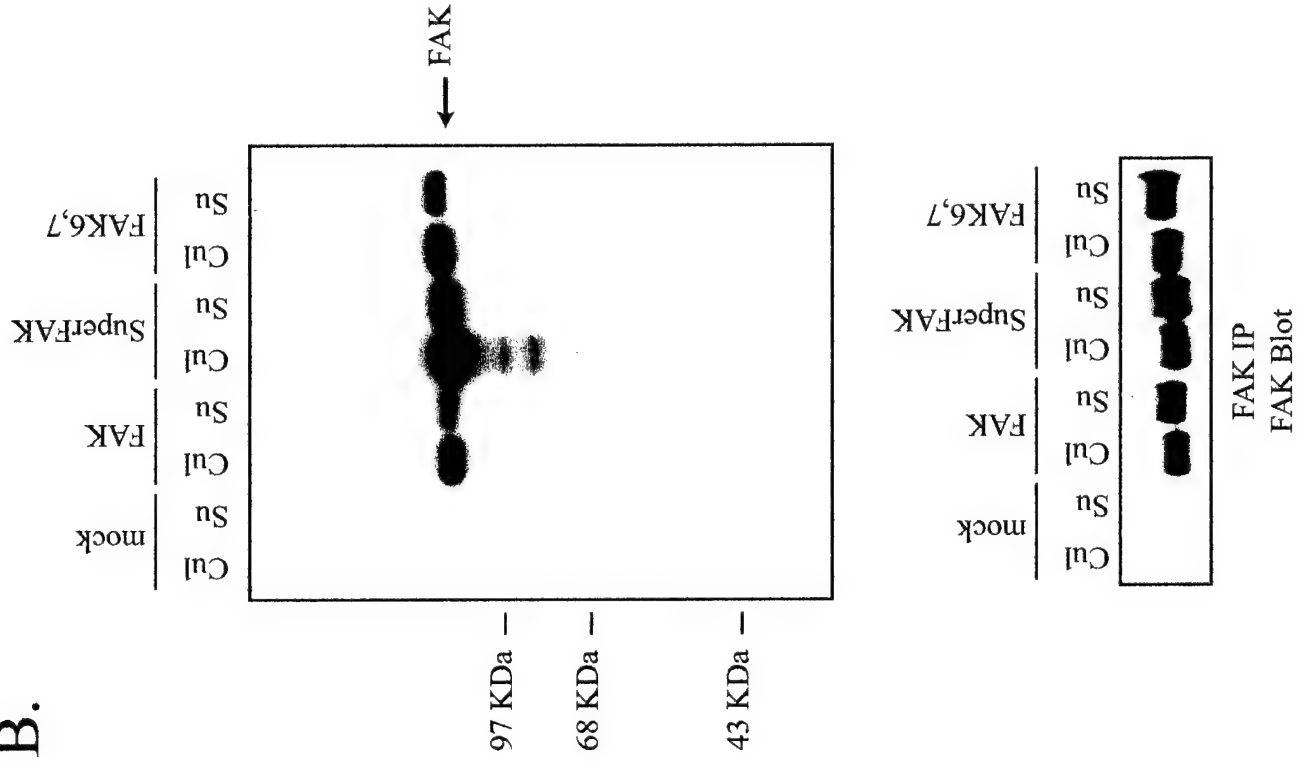


Figure 5

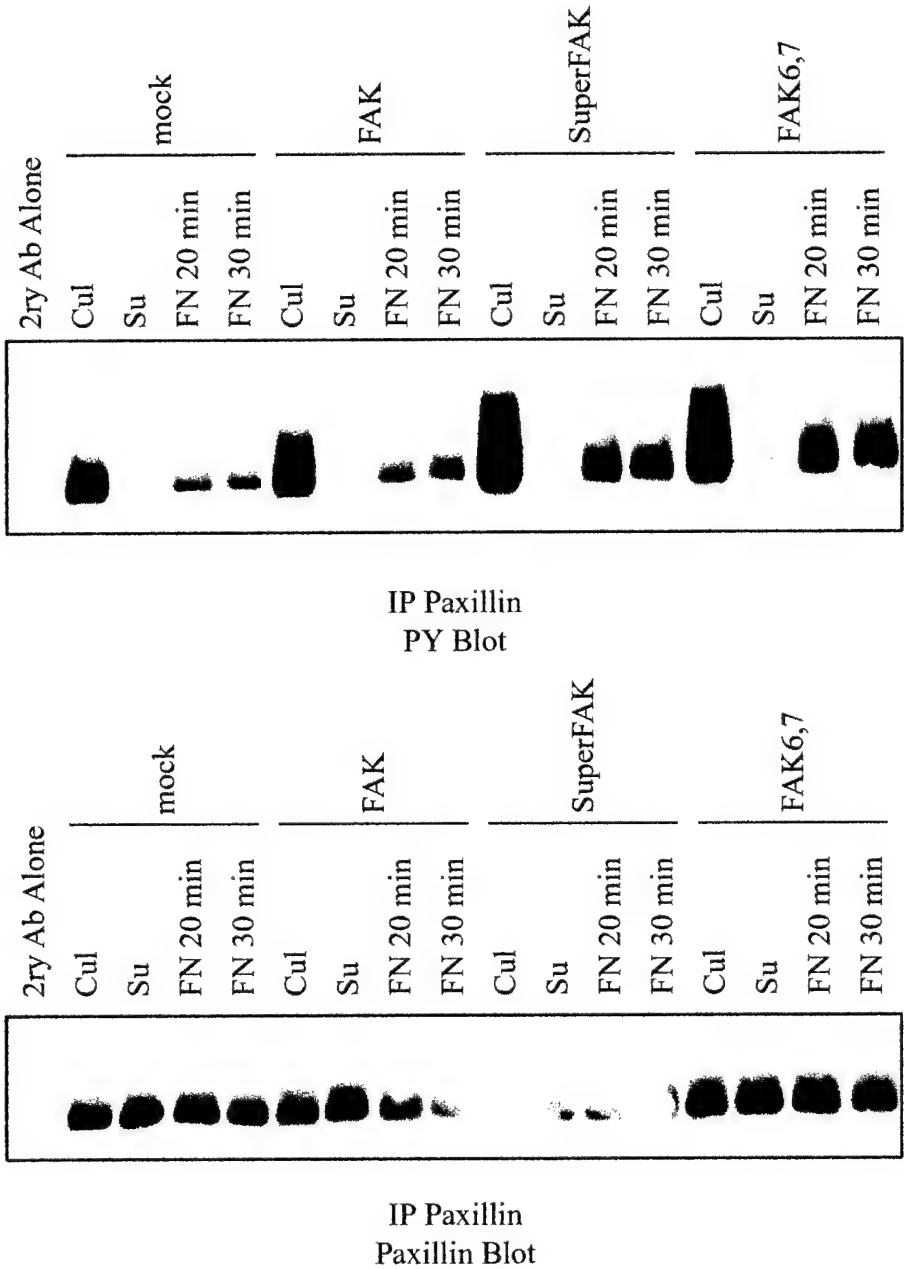
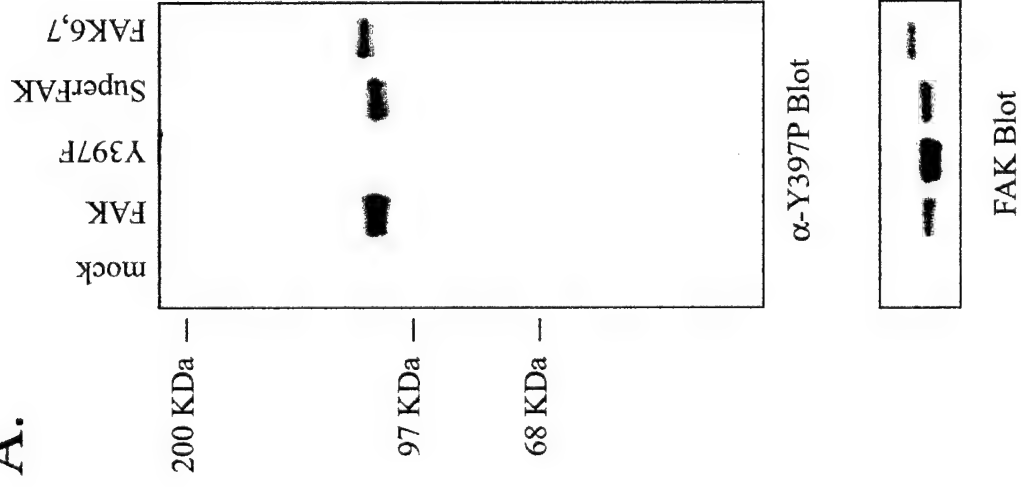
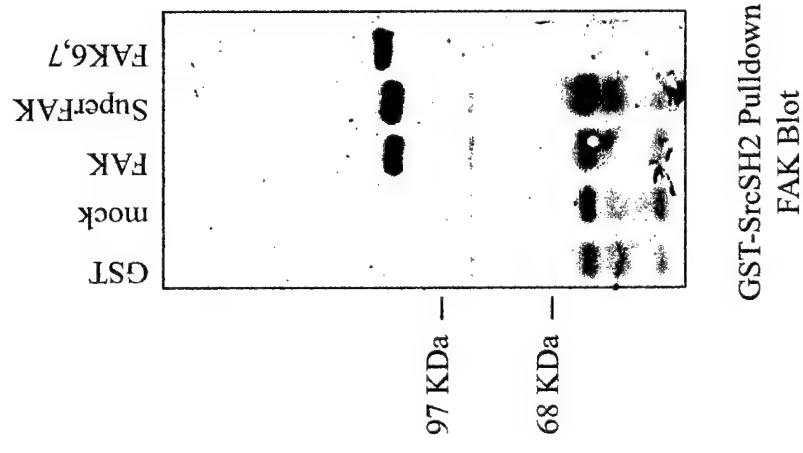


Figure 6

A.



B.



C.

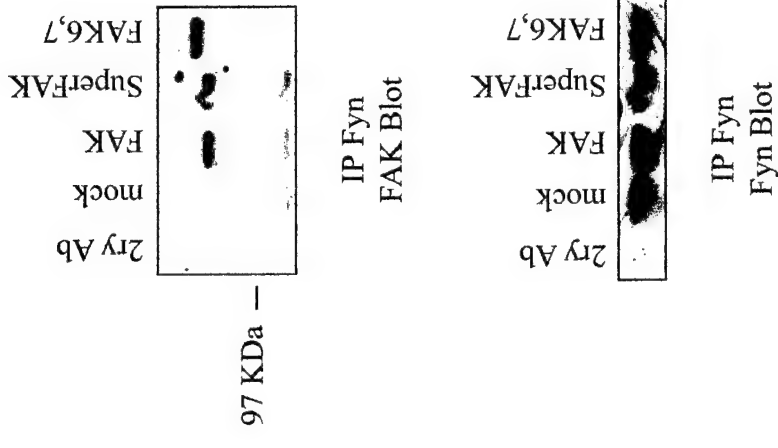


Figure 7

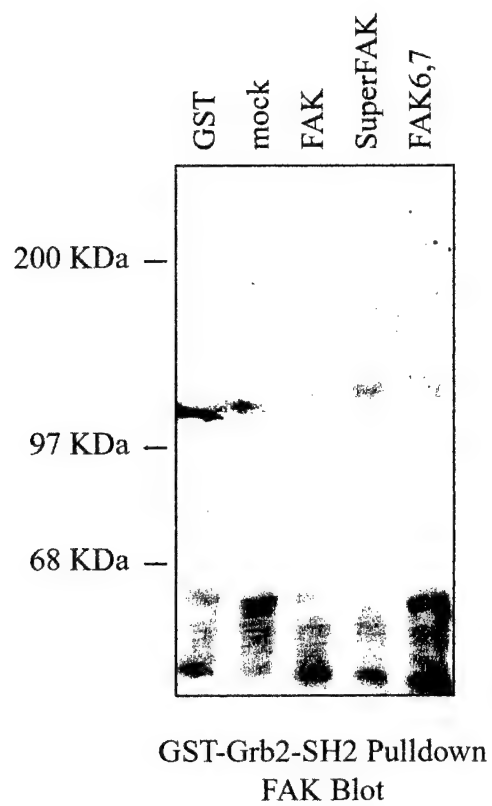
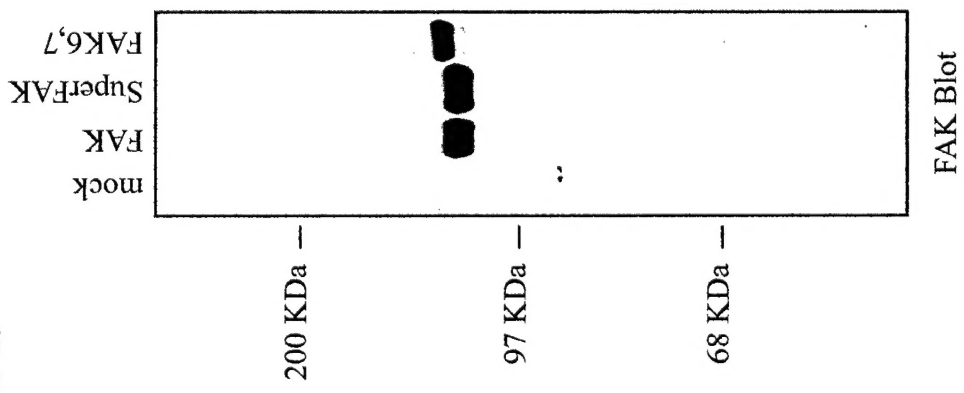
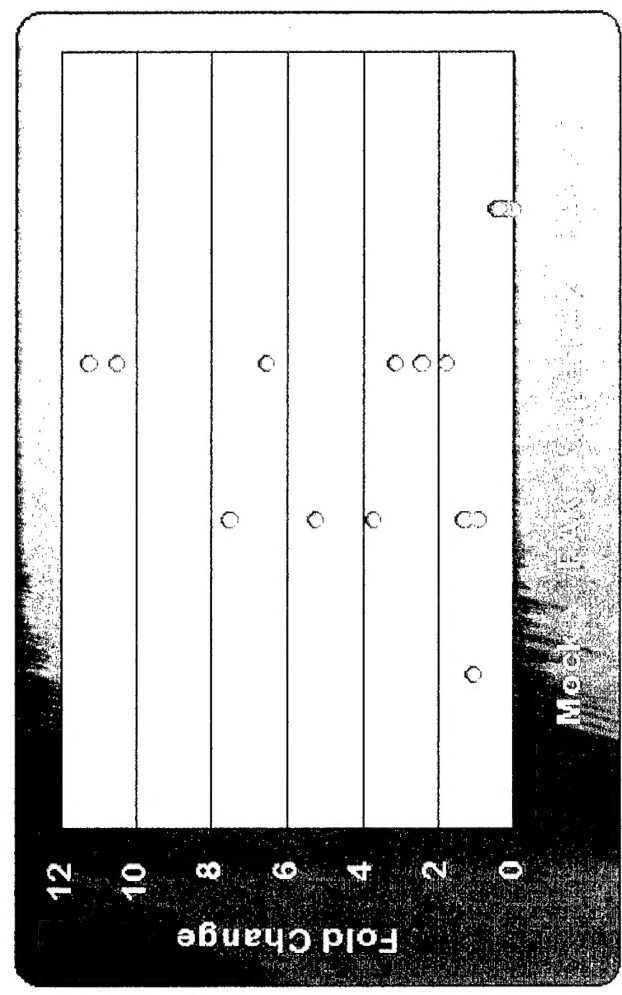


Figure 8

A.



B.



APPENDIX C

Keystone Meeting Abstract
"Signaling 2000"
Keystone Colorado
January 22-28, 2000

Characterization of a Hyperactive Mutant of FAK

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FAK is a non-receptor tyrosine kinase found to play an important role in normal biological processes such as adhesion, spreading, migration, motility, cell cycle, and cell survival. In addition, FAK is overexpressed in a variety of cancer cells and tumors. In order to further investigate the biological roles of FAK, constitutively activated forms of FAK were designed. Targeting a CD2/FAK chimera to the membrane has been reported to lead to constitutive FAK activation. However, FAK is not membrane localized and thus the CD2/FAK chimera may not accurately mimic FAK signaling. Our goal was to build constitutively active mutants of FAK without altering their cellular localization. An alternatively spliced form of FAK in rat brain has been reported, which is characterized by having a high autophosphorylation activity. The presence of two additional exons flanking the autophosphorylation site (Y397) was sufficient to increase FAK's activity. The brain variant was re-engineered by oligonucleotide directed mutagenesis (FAK6, 7). Based on reports that the K650E mutation of the fibroblast growth factor receptor 3 (FGFR3) increases the kinase activity, the two lysines in the activation loop of FAK were mutated to glutamic acids (KKEE). KKEE, and to a lesser degree FAK6,7, exhibited increased catalytic activity *in vitro*. The expression of FAK6,7 or KKEE in chicken embryo fibroblasts, caused an increase in the phosphotyrosine level of tensin, FAK and paxillin. The phosphotyrosine increase was noticeably higher with KKEE. However, the effect on the phosphorylation of FAK substrates was adhesion dependent. In future studies, KKEE, a hyperactive mutant of FAK, will be expressed in MCF10A and T47D cells to further investigate the role of FAK signaling in cancer progression and oncogenesis.

Veronica Gabarra
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Meeting B1
Poster Session 1

APPENDIX D

American Society for Cell Biology 40th Annual Meeting
San Francisco, CA
December 9-13, 2000

Characterization of a Hyperactive Mutant of FAK

Focal Adhesion Kinase (FAK) is a non-receptor tyrosine kinase found to play an important role in normal biological processes such as adhesion, spreading, migration, motility, cell cycle, and cell survival. In addition, FAK is overexpressed in a variety of cancer cells and tumors. In order to further investigate the biological roles of FAK, constitutively activated forms of FAK were designed. Targeting a CD2/FAK chimera to the membrane has been reported to lead to constitutive FAK activation. However, FAK is not membrane localized and thus the CD2/FAK chimera may not accurately mimic FAK signaling. Our goal was to build constitutively active mutants of FAK without altering their cellular localization. An alternatively spliced form of FAK in rat brain has been reported, which is characterized by having a high autophosphorylation activity. The presence of two additional exons flanking the autophosphorylation site (Y397) was sufficient to increase FAK activity. The brain variant was re-engineered by oligonucleotide directed mutagenesis (FAK6.7). Based on reports that the K650E mutation of the fibroblast growth factor receptor 3 (FGFR3) increases the kinase activity, the two lysines in the activation loop of FAK were mutated to glutamic acids (SuperFAK). SuperFAK, and to a lesser degree FAK6.7, exhibited increased catalytic activity *in vitro*. The expression of FAK6.7 or SuperFAK in chicken embryo fibroblasts, caused an increase in the phosphotyrosine level of tensin, FAK and paxillin, which was noticeably higher with SuperFAK. However, the effect on the phosphorylation of FAK substrates was adhesion dependent. SuperFAK caused an elevation in the ability of breast epithelial cells, T47D, to migrate in response to a haptotactic signal. In future studies, SuperFAK, a hyperactive mutant of FAK, will be expressed in breast epithelial cells to investigate the role of FAK signaling in the acquisition of a cancer phenotype(s).